JUL 31 1923

Vol. XVII, No. 3 1923

Medical Lib.

THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

SIR W. M. BAYLISS, F.R.S.

AND

ARTHUR HARDEN, F.R.S.

EDITORIAL COMMITTEE

PROF. G. BARGER
PROF. V. H. BLACKMAN
MR J. A. GARDNER
PROF. F. G. HOPKINS
SIR F. KEEBLE
PROF. W. RAMSDEN
SIR E. J. RUSSELL

CAMBRIDGE UNIVERSITY PRESS
C. F. CLAY, Manager
LONDON: Fetter Lane, E.C. 4
also
H. K. LEWIS & Co., Ltd., 136, Gower Street, London, W.C. 1
WHELDON AND WESLEY, Ltd., 2–4, Arthur Street, New Oxford Street, W.C. 2
CHICAGO: The University of Chicago Press
(Agent for the United States and Canada)
BOMBAY, CALCUTTA, MADRAS: Macmillan and Co., Ltd.
TOKYO: The Maruzen-Kabushiki-Kaisha

1923

Price Twenty Shillings net

PRINTED IN GREAT BRITAIN

The *Biochemical Journal* is conducted by the Biochemical Society and is published by the Cambridge University Press.

This Society has been instituted for the purpose of facilitating intercourse between those biologists and chemists who are interested in the investigation of problems common to both, such as the chemical problems connected with Agriculture, Brewing, Animal and Vegetable Physiology and Pathology, &c. Persons interested in Biochemistry are eligible for election.

Meetings are held at different centres for the communication of papers to the Society.

The annual subscription is 35*s*., which includes a copy of the Journal, and becomes due on January 1st. Further information may be obtained on application to the Hon. Sec., Dr H. W. Dudley, National Institute for Medical Research, Mount Vernon, Hampstead, London, N.W.3, or to the Hon. Treas. Mr J. Addyman Gardner, 17 Brunswick Gardens, Kensington, W. 8, to whom subscriptions should be sent.

Papers for publication should be sent to Sir W. M. Bayliss, F.R.S., University College, W.C. 1, or to Prof. A. Harden, F.R.S., Lister Institute, Chelsea Gardens, S.W. 1. Communications respecting the printing of the articles, or respecting the purchase of offprints should be addressed to the University Press, Cambridge.

The Journal is issued about every two months and the date at which each paper is received by the editors is printed at the beginning of the paper.

All communications respecting the purchase of copies of the parts or volumes, whether current or back issues, or respecting subscriptions in the case of non-members of the Biochemical Society (£3 net per volume (post free) payable in advance) should be addressed to Mr C. F. Clay, Cambridge University Press, Fetter Lane, London, E.C. 4, or to the Hon. Treas. Mr J. Addyman Gardner, 17 Brunswick Gardens, Kensington, W. 8. For prices of back numbers and volumes see p. 4 of this wrapper.

Quotations can be given for Buckram binding cases and for binding Subscribers' Sets.

The Cambridge University Press has appointed the University of Chicago Press agent for the sale of the *Biochemical Journal* in the United States of America and has authorised the following subscription price. $14.25 net.

Notice to Contributors.

1. Contributors receive 25 copies of their papers free and can purchase additional copies if notice be given when their proofs are returned.

2. Papers forwarded to the Editors for publication are understood not to be offered to any other Journal for prior or simultaneous publication.

3. All communications intended for publication must be type-written.

4. **References.** References to literature should be arranged alphabetically according to authors' names at the end of the communication, each accompanied by the date, title of Journal, volume and page, thus: Ackermann (1912, 1), *Zeitsch. Biol.* 59, 17. In the text the reference should be indicated by the author's name and the date, enclosed in brackets, thus [Ackermann, 1912, 1]. If reference is made to several papers published by one author in a single year, these should be numbered in sequence and the number quoted along with the year both in the text and in the collected references.

5. Chemical formulae should be written, as far as possible, in a single horizontal line.

6. Illustrations and curves accompanying the papers must be carefully drawn, about twice the size of the finished block, on smooth white Bristol boards in Indian ink. Any lettering on these drawings should be lightly inserted in pencil. Further information can be obtained from the Editors.

XLI. THE RELATION OF CERTAIN BLOOD CONSTITUENTS TO A DEFICIENT DIET.

By T. J. THOMPSON and IDA L. CARR.

From the Biochemical Division of the Department of Chemistry, University of Nebraska, Lincoln, Nebraska.

(*Received April 2nd, 1923.*)

The purpose of this investigation was to determine the variation of certain blood constituents with the development of polyneuritis in chickens, a subject on which, as far as we know, no work has been done.

Mature Plymouth rock hens were used for the experiment. They were given a normal ration for a short time to get them as near as possible to a normal condition. They were then fed on boiled polished rice, with filter paper added to give bulk, and crushed marble for grits.

In addition to the loss of appetite and the decrease in body weight observed by Anderson and Kulp [1922] the hens developed a craving for other foods, and would eat straw and peck at the clothing of the attendants. They were laying at first, but as the experiment progressed, there was a gradual decrease in the number of eggs produced until finally they stopped laying. The eggs had a "puckery" taste and the yolks became light coloured and smaller. Feathers did not develop where they had been plucked, when the blood was drawn. As the rice feeding continued there was a drop in temperature from 1 to 2° varying among the different chickens.

The blood was drawn from the brachial artery under the wing with a hypodermic needle, and was collected in a graduated cylinder. The needle and graduated cylinder were previously rinsed with kerosene, and potassium oxalate sufficient to prevent clotting was added. 10 cc. of blood was drawn at a time, the loss of which apparently did not affect the chickens.

Chicken blood clots more quickly than human or ox blood. There is also a wide variation among individual chickens in the tendency to clot. With some it was always difficult to secure samples; with others it was very easy. Toward the end of the rice feeding period, it became increasingly difficult in every case; and as the polyneuritis symptoms developed, it became impossible to obtain samples. The number of punctures made during the drawing of a single sample increases the tendency to clot very markedly.

Determinations of sugar, uric acid, creatinine and total non-protein nitrogen were made, using the Folin-Wu method [1919] of blood analysis (see Table I). Ogata and associates [1921] found an abnormal amount of sugar in

the blood of pigeons suffering from polyneuritis. An examination of Table I shows that in some cases the final value for sugar was greater than normal. In one individual there was a regular increase in the amount of sugar. The non-protein nitrogen values were somewhat erratic. The uric acid and the creatinine values showed the most regular variation, decreasing at first then suddenly increasing. Soon after this increase occurred, the hen showed signs of polyneuritis. The increased uric acid was apparent from two to five weeks before paralysis set in. Autopsies of the chickens which died showed deterioration of the heart, kidneys, or liver. This disintegration of the solid organs was probably responsible for the increase in the amounts of uric acid and creatinine with the onset of polyneuritis.

Table I. *Results are given in milligrams per* 100 *cc. of blood.*

Hen number	Days on diet	Non-protein nitrogen	Creatinine	Sugar	Uric acid	Weight lbs.	Weight oz.	Remarks
1	0	45·29	1·09	176·56	5·10	5	7	After 87 days paralysis developed. Recovered on yeast-rice diet. In 121 days xerophthalmia[1] developed. Died 9 days later.
	24	42·38	1·38	269·36	8·65	5	8	
	40	39·58	1·33	174·84	4·47	5	5	
	55	40·93	1·20	228·83	5·31	5	4	
	69	33·38	1·41	217·08	5·53	4	4	
2	0	35·68	1·03	237·68	5·89	—		Paralysis developed in 86 days. Put on yeast-rice diet and recovered.
	10	37·53	1·19	199·67	4·96	7		
	44	51·81	1·08	263·99	5·68	6	12	
	60	41·44	1·22	198·90	7·05	6	5	
3	0	41·80	2·02	176·98	4·75	6		Paralysis appeared in 74 days. Recovered on rice-yeast diet.
	10	37·20	1·38	178·06	3·98	6		
	33	44·58	1·35	180·60	1·99	5	12	
	44	42·60	0·97	254·48	7·61	5	1	
4	0	36·34	1·45	229·30	2·90	7		Had paralysis in 80 days. Died. Autopsy revealed degenerated liver.
	33	40·70	1·35	198·25	2·05	6	5	
	44	25·94	1·03	229·57	7·23	6	6	
5	0	42·48	1·46	214·70	2·24	5	12	Last sample taken 2 days after paralysis occurred. Died. No organic disturbance shown on autopsy.
moulting	25	40·22	1·66	221·84	13·01	3	14	
6	4	36·43	1·62	248·62	6·50	6	7	Paralysis appeared in 28 days. Died in 37 days. Heart and kidneys degenerated.
moulting	22	45·99	2·25	241·43	13·98	5	4	
7	0	37·51	1·51	239·34	8·56	6	12	Developed paralysis in 45 days. Recovered on yeast-rice diet.
moulting	42	32·36	1·36	238·65	9·50	4	11	
8	0	34·44	1·60	218·19	1·98	5	3	Two days after last sample was taken, hen died. Autopsy showed an oedema between leg and body and degenerated liver.
moulting	22	23·73	2·48	198·64	2·29	5		
	28	59·51	3·10	268·63	7·51	4	9	

[1] [McCollum and Simmonds, 1917.]

When the hens became so paralysed as to be unable to walk about they were fed 30 g. yeast per day and as much boiled rice as they would eat. They showed marked improvement at once although they did not gain much in weight after the first day. The hens which ate the yeast rice mixture voluntarily improved more rapidly than those which were forcibly fed. All of them were able to stand and walk about after a few days. About a week later while still on the rice yeast diet they began to lay again, and continued laying an egg every week or ten days until the end of the experiment.

SUMMARY.

1. A method for drawing blood is given by which the chickens are not injured.

2. The tendency of the blood to clot varies widely in different chickens and increases with the number of punctures during a single blood-letting.

3. There is a marked increase in the uric acid and creatinine content of the blood with the onset of polyneuritis.

4. Autopsy showed degeneration of the solid organs of the chickens upon which the polyneuritis was allowed to run its course.

5. Chickens which were fed on yeast after they developed polyneuritis showed marked increase in vitality, although there was very little increase in weight.

6. The variation of the two groups of chickens with reference to the development of polyneuritis shows that such experiments should be so regulated that the moulting season is avoided. (See appended table.)

REFERENCES.

Anderson and Kulp (1922). *J. Biol. Chem* **52**, 69.
Folin and Wu (1919). *J. Biol. Chem.* **38**, 81.
McCollum and Simmonds (1917). *J. Biol. Chem.* **32**, 29
Ogata, Kawakita, Oka, Kagoshima and Nasshin (1921). *Prog. Med.*; Abs. in *Exp. Sta. Rec.* (1922), No. 6, 46; No. 7, 658.

XLII. THE PURIFICATION OF INSULIN AND SOME OF ITS PROPERTIES.

By HAROLD WARD DUDLEY.

Department of Biochemistry and Pharmacology, National Institute for Medical Research.

(*Received April 24th, 1923.*)

The discovery of Banting and Best [1922] of the pancreatic hormone, insulin, which affects the metabolism of the normal animal so profoundly, and which has proved to be of such great practical importance in the treatment of human diabetes, has aroused widespread interest.

The present communication describes attempts to obtain the active principle in as pure a condition as possible, and experiments which have been made with the hope of determining some of its general chemical characteristics.

The insulin employed in this research has been made from fresh ox pancreas by a method which is essentially that of Collip [1923] with certain slight modifications which we have found advantageous, and is described in detail in the experimental section of this paper. The active fraction, which we may call "crude insulin," is obtained in the form of a white, hygroscopic powder, very readily soluble in water, in which it dissolves to form a pale straw-coloured solution which is slightly acid to litmus. The yield varies considerably from preparation to preparation, but the average yield from 21 batches, in which a total weight of 103·6 kilos. pancreas was worked up, was 0·718 g. per kilo. The estimation of the activity of these preparations has been based on the original "Toronto rabbit-unit," *i.e.* the dose necessary, when injected subcutaneously, to lower the blood sugar of a 2 kilo. rabbit, which has fasted for 16–24 hours, from a normal value of about 0·1 % to about 0·04 % and to cause typical hypoglycaemic convulsions within four hours.

Our own experience confirms the observations of McCormick, Macleod, Noble and O'Brien [1923] that the rabbit test is by no means quantitative. We find that rabbits, even after the same preliminary preparation by starving, vary greatly in sensitiveness to similar doses of insulin. The same rabbit will usually, though certainly not always, suffer about the same fall in blood sugar whenever it is given identical doses of insulin.

It is, therefore, possible, by using large numbers of animals, and particularly animals whose reactions are well-known from repeated experiments, to form a fairly accurate opinion as to the relative activities of various insulin preparations, though a really quantitative method of estimation is not yet available.

The dose of "crude insulin" necessary to cause hypoglycaemic convulsions in a 2 kilo. rabbit has been, in our most active preparations, 5 mg. or less, but more usually it is about 10 mg., and we get, on an average, a "rabbit unit" from about 15 g. pancreas.

Over 50 % of "crude insulin" consists of inorganic salts, and it gives positive reactions for chlorides and phosphates.

The following reactions are also positive: biuret, Millon (tyrosine), glyoxylic acid (tryptophan), Pauly (iminazole ring), Molisch (carbohydrate) and Seliwanoff (fructose). A solution in caustic soda, to which lead acetate has been added, blackens on heating, indicating the presence of sulphur.

It is obviously a very complex mixture, and it is impossible to associate any of its reactions with the physiologically active material.

The hormone is precipitated from an aqueous solution of "crude insulin" by a variety of reagents, such as uranium acetate and phosphotungstic acid, but the most practically useful precipitant is picric acid, which removes it from dilute aqueous solution quantitatively. The picrate, which is an amorphous, lemon-yellow powder, weighs from one-twelfth to one-fourteenth of the amount of crude insulin taken. It is only very slightly soluble in water but can be dissolved in dilute ($N/15$) di-sodium hydrogen phosphate solution for testing on rabbits. A rabbit unit of the picrate is about 1 mg.

It has been found to be quite easy to convert this insoluble picrate into a soluble hydrochloride. It is ground up with a little absolute alcohol until a uniformly fine suspension is obtained. A small quantity of an alcoholic solution of hydrochloric acid is then added and the mixture is stirred for a few minutes and dry ether is finally added. The hydrochloride separates out as an almost white powder which may be washed free from picric acid with dry ether. It is a white, perfectly non-hygroscopic and stable powder, readily soluble in water, and is obtained in a yield of 75–80 % of the picrate. The rabbit unit of this material falls generally between 0·5 and 1 mg.

The yield of hydrochloride is therefore 1 g. from 20–25 kilos. pancreas and approximately 6 % of the "crude insulin." Within the limits of determination possible by the admittedly inaccurate rabbit test, we have not been able to detect any loss of activity in preparing the hydrochloride from "crude insulin."

"Crude insulin" contains some material which causes a moderate, though transient, fall in the blood pressure of an anaesthetised animal. This substance is completely eliminated in the purification effected by means of picric acid, and the hydrochloride consequently displays no such effect.

The chemical reactions of the hydrochloride are interesting; in contrast to "crude insulin" its solution no longer gives an immediate positive test for phosphate. Further, after oxidising the solid with a mixture of nitric and sulphuric acids the test for phosphoric acid is negative, and indicates the absence of organically combined phosphorus.

This confirms the observation of Doisy, Somogyi and Shaffer [1923] made on "insulin" purified by a different procedure. Moreover, it no longer gives

Seliwanoff's reaction for fructose. This therefore disposes of the suggestion of Winter and Smith [1923] that these reactions may be associated with the active principle.

It no longer gives any reaction for tryptophan, and the Millon reaction (tyrosine) is now very faint and atypical. It still gives a positive Molisch (carbohydrate) reaction, but it is well-known that this reaction is almost always present in protein-containing material. The biuret reaction is given strongly, the Pauly reaction is intense, and the organic sulphur reaction is quite marked. It is, in fact, a substance, exhibiting the characteristics of a protein derivative, in which, of the common tests applied in such cases, the biuret and those for the iminazole ring (histidine) and for sulphur are the only striking reactions. It should be noted that Best and Macleod [1923] state that insulin prepared from the ox and pig pancreas gives a biuret reaction, an orange colour by the xanthoproteic test and a faint purple with glyoxylic acid. They fail, however, to produce any of these reactions with insulin prepared from skate pancreas by alcoholic extraction and heat. Doisy, Somogyi and Shaffer attribute a distinct biuret reaction, faint glyoxylic acid and doubtful Millon reactions to their purest preparation.

None of these investigators appears to have tried the Pauly and sulphur tests which are the characteristic reactions of the preparation from ox pancreas; the others, with the exception of the biuret, vanish or become insignificant when insulin is purified by precipitation with picric acid. It may be mentioned, in passing, that small amounts of insulin were prepared in the course of this investigation from the pancreas of the dog-fish and of the skate by the method described in this paper, and in both cases the "crude insulin" gave a very definite biuret reaction.

Whether the sulphur is present as cystine, or as some other organic sulphur-containing compound, is at present uncertain. After hydrolysis with hydrochloric acid under suitable conditions a solution is obtained which, after reduction, gives the sodium nitroprusside reaction, carried out according to Hopkins' instructions [1921], with considerable intensity. Hydrolysis with stronger acid, however, apparently destroys the substance which gives this reaction. Further experiments are being performed in this connection. It is impossible to obtain a positive nitroprusside reaction in a reduced solution of insulin hydrochloride itself. The sulphur in the intact substance is therefore not reducible, as it is, for example, in glutathione.

Doisy, Somogyi and Shaffer [1923] have shown that insulin may be precipitated from aqueous solution at what is called its "isoelectric point" by adjusting the p_H of the solution to about 5. If a solution of the hydrochloride be treated in this manner a precipitate is formed which has a rabbit unit value of about 0·3 mg.

The whole of the active substance, however, is not precipitated, at any rate from a 2 % solution of the hydrochloride. This precipitate, like the hydrochloride, gives strong Pauly and sulphur reactions.

The hydrochloride dissolves in water giving a solution with a p_H of about 4, and the "isoelectric point" precipitate is produced by adding alkali to such a solution. A precipitate is also formed by adding strong acid to the hydrochloride solution, until it contains about 3·3 % HCl. A flocculent white precipitate is formed which is somewhat more active than the "isoelectric point" precipitate. 0·25 mg. of this substance proved to be a rabbit unit, and it is the most potent preparation so far obtained.

In this case also the precipitation of the active principle is not complete. The precipitate, again like the hydrochloride, gives strong Pauly and sulphur reactions.

Experiments have been made to ascertain the stability of insulin to acid and alkali. It proves to be rather surprisingly stable to acid. If an aqueous solution of "crude insulin" is heated to 100° for 10 minutes in a boiling water-bath a great deal of its activity is destroyed. But in $N/10$ H_2SO_4 it will stand this treatment for 30 minutes without detectable loss of activity. After one hour of such treatment it is obvious that some destruction has taken place, and still more after two hours, but even then a considerable amount of the active principle remains.

Very different is its behaviour to alkali. Heating to 37° for $1\frac{1}{2}$ hours in $N/10$ NaOH destroys its activity completely, and the same destruction is effected by heating to 100° in a boiling water-bath for 10 minutes in $N/10$ Na_2CO_3. It is more slowly inactivated by digestion in $N/10$ Na_2CO_3 at 37°, retaining a considerable amount of activity after two hours under these conditions.

It is noteworthy that not only is "insulin" readily destroyed by trypsin, thus confirming the original supposition of the Toronto workers, on which they based their methods for its isolation, but it is also decomposed with remarkable ease and rapidity by pepsin.

An attempt to filter a solution of "crude insulin" through a collodion sack under pressure showed that only minute amounts of the active principle passed through.

As before mentioned, addition of uranium acetate to a solution of crude insulin precipitates the active principle completely. A point of some practical importance was established when it was found that the insulin could be washed off the precipitate by means of weakly alkaline solutions of disodium hydrogen phosphate. It is evidently adsorbed on the precipitate of uranyl phosphate in virtue of its colloidal nature. For human use insulin has to be sterilised by filtration through Berkefeld filters, and serious losses of activity had been experienced when solutions of "crude insulin" were so filtered. These solutions are faintly acid, with a p_H not far removed from the "isoelectric point." In consequence of the uranium acetate experiment it was found that if the p_H of a solution of insulin is first adjusted to approximately 7·5 it may be filtered through a candle without detectable loss. It is obvious that filtration of acid solutions is bound to be a risky procedure in view of

the formation of precipitates, not only at the "isoelectric point," but also in stronger acid.

A review of the results obtained enables us to draw certain general conclusions.

The *complete* precipitation of insulin from dilute solution by picric acid, its ready adsorption, its failure to pass through an ultra-filter, its rapid digestion by trypsin, and particularly by pepsin, all indicate that it is most probably a substance having a very complex structure and protein-like in its nature. The hope of its isolation as a chemically pure substance becomes slender, and of its synthesis very remote, by methods at present at our disposal.

It is certainly much more complex than the active principles of the pituitary posterior lobe, probably even more so than "secretin." It is more analogous in complexity to the toxins, to ricin, or to Vaughan's toxic protein derivatives.

It is obvious that the administration of insulin to patients by the mouth is impossible on account of its rapid destruction by the digestive enzymes, and the relatively large size of its molecule makes it unlikely that absorption from mucous surfaces, such as that of the nasal passages, a method which has recently proved useful in controlling diabetes insipidus with pituitrin, will be practically useful in the case of insulin.

The fact that great physiological activity is associated with both the "isoelectric point" precipitate and the strong hydrochloric acid precipitate, described in this paper, suggests that the active principle is present in such a preparation as the "insulin hydrochloride" in quite indeterminate amount, associated with other substances of approximately similar physical properties. In this case it is impossible to decide whether the characteristic Pauly and sulphur reactions bear any relation to the actual active principle. It is, however, intriguingly interesting that the Pauly reaction should be so strongly positive. It occurs so frequently in connection with preparations of highly physiologically potent substances, such as those of the oxytocic and pressor principles of the pituitary gland, that one is tempted to suspect that derivatives of histidine are apt to be endowed with various intense physiological activities; but, at present, this is pure speculation.

Experimental.

Preparation of "Crude Insulin."

Five kilos. of perfectly fresh ox pancreas are minced through a sausage machine into 5 litres of 95 % alcohol, previously cooled to $-3°$. Not more than two hours should elapse between the slaughtering of the animals and the mincing of the tissue into alcohol. It is advantageous to strain off the minced tissue on a fine sieve, squeeze out as much of the alcohol as possible by hand, remince the tissue into the alcohol and repeat the mincing a third time in order to get as good

disintegration as possible. The mixture is then allowed to stand for two hours at room temperature with frequent stirring. It is then filtered through large folded filters of thick "Chardin" paper. As soon as the main bulk of the liquid, which may be slightly turbid, is filtered, the thick paste on the paper is transferred to a suitable press and as much liquid as possible is squeezed out. The press liquor and filtrate are mixed. This filtration is tedious, but is completed in three to four hours. Approximately 5·5 litres of filtrate are collected. $1\frac{1}{2}$ volumes (*i.e.* 8·25 litres) of 95 % alcohol are now added. A white precipitate appears and the liquid is placed in a cold room at − 3° overnight.

The liquid is then filtered through "Chardin" folded filters in the cold room. This filtration proceeds without difficulty, giving a perfectly clear, yellow solution. The filtrate is transferred in quantities of $2\frac{1}{2}$–3 litres to a battery of five 5-litre round-bottomed flasks, and the alcohol is distilled off *in vacuo*, the temperature of the water-baths being kept at 45°.

When most of the alcohol has been removed the liquid becomes turbid owing to the separation of fat. The residual liquid in the five flasks is collected in one flask and further distilled *in vacuo* with the water-bath at 45°. This removes the remainder of the alcohol, and the fat usually separates in buttery lumps. The distillation is continued until the volume of the aqueous residue is about 250 cc. It is then filtered from fat on a Buchner funnel and the main bulk of the fat is shaken out of the flask and squeezed as free from solution as possible. (Occasionally the fat does not solidify but remains floating in the concentrated aqueous solution as a thick, yellow oil. When this happens, the entire contents of the flask are transferred to a separating funnel and shaken with a small quantity of light petroleum. This dissolves the fat readily, and the aqueous layer can be tapped off without loss.) The aqueous solution is then made up to 80 % with absolute alcohol; that is to say, to every 100 cc. of solution are added 400 cc. of absolute alcohol. A yellow syrup separates in fine drops and falls to the bottom of the solution which is then placed in the cold room overnight.

After standing, the syrup and a certain amount of solid matter adhere to the bottom of the containing vessel, preferably a tall, narrow cylinder, and the supernatant liquid is decanted without difficulty and made up to 93 % by the addition of two volumes of absolute alcohol. A somewhat scanty white precipitate forms and the liquid is placed in the cold room for 15–20 hours. The precipitate has now settled to the bottom as a white granular powder, the supernatant liquid being almost, if not quite, clear. The latter is removed by means of a siphon except for a small amount left covering the precipitate, which is then stirred up with the residual alcohol and poured into centrifuge tubes. The precipitate is centrifuged down, washed once or twice with absolute alcohol and once or twice with dry ether in the centrifuge. After pouring off the ether the tubes containing the precipitate are quickly transferred to a vacuum desiccator and dried over sulphuric acid. A yield of from 3–4 g. is obtained in the form of a white, hygroscopic powder.

Preparation of the Active Picrate and Hydrochloride.

A 1·5 % aqueous solution of "crude insulin" is made. It is freed from any small amount of suspended matter by centrifuging—filtration, even through paper, is apt to be attended by loss of activity—and then half its volume of a saturated aqueous solution of picric acid is added. An immediate flocculent, amorphous precipitate is formed which settles down rapidly to the bottom of the liquid, leaving a fine suspension in the supernatant fluid. After standing for one or two days at ordinary room temperature the supernatant liquid becomes quite clear and is poured off. A little water is then added to the precipitate which is now adhering to the bottom of the beaker. The picrate is rubbed up with a glass rod and the suspension is then transferred to a centrifuge tube and spun down. It is washed with repeated additions of water in the centrifuge until finally the supernatant liquid displays a slight turbidity on the addition of a few drops of saturated aqueous picric acid. This indicates that the picrate is beginning to go into solution. It is then suspended in a small volume of water and filtered on a small Buchner funnel and finally placed on porous pot and dried in a vacuum desiccator over H_2SO_4. A lemon-yellow amorphous powder is obtained in a yield of about 7·5 % of the crude insulin taken.

The precipitation should be carried out in dilute solution as indicated, although it is permissible to use a 2 % solution of "crude insulin," adding equivalently larger amounts of picric acid solution. If precipitation from strong solutions is attempted other picrates are precipitated also which are inert physiologically.

The mother liquor from the precipitation of the picrate has been examined in numerous batches for blood-sugar lowering material. It has been evaporated *in vacuo* to a convenient bulk, then faintly acidified with hydrochloric acid, the picric acid shaken out with ether, and finally injected into a rabbit. We have never succeeded in finding a trace of insulin in this way. For instance, 1 g. of "crude insulin," which had a rabbit unit of 10 mg. was precipitated with picric acid in the manner described. Half the filtrate, representing 0·5 g. of the original powder, or 50 rabbit units, was concentrated *in vacuo* to about 10 cc. It was then acidified with 2 cc. *N* H_2SO_4 and the picric acid was removed by shaking out with ether. The ether was removed from the solution *in vacuo* and then, after adding 2 cc. *N* Na_2CO_3, the whole solution was injected into a 2·4 kilo. rabbit subcutaneously. The blood sugar values, taken every hour, were as follows:

Blood sugar, normal	0·096 %
,, ,, 1 hour after injection	0·093
,, ,, 2 hours ,, ,,	0·101
,, ,, 3 ,, ,, ,,	0·105

It will be seen that there is no fall in the blood sugar value, indicating that the removal of insulin by means of picric acid is quantitative.

The proper washing of the picrate is of great importance. It is more practicable to do the washing in a centrifuge tube since the picrate tends to clog on a filter paper. If any mother liquor remains in the wet precipitate it will be found that, on drying, the picrate, instead of remaining pale yellow and easily powderable, forms a hard, dark brown mass, rather like shellac. In this condition it is quite unsuitable for conversion into the hydrochloride. If a batch of picrate is found in this state after drying it should be dissolved in $N/10$ Na_2CO_3 and the clear solution should then be acidified with HCl until just acid to litmus. After the addition of a small amount of picric acid solution the reprecipitated picrate is washed thoroughly and recovered as described above. It may be noted that on reprecipitation in this manner the picrate is in a somewhat more easily filterable condition and may be collected and washed on a filter paper.

If a sufficient quantity of picrate is available this reprecipitation is to be recommended as giving a slightly purer product, which is also in better physical condition for the subsequent transformation into hydrochloride.

This is accomplished by grinding up 1 g. of picrate with 6 cc. absolute alcohol in a mortar until a very fine uniform suspension is obtained. It is then transferred to a centrifuge tube of about 40 cc. capacity and washed in with 2 cc. absolute alcohol. 4·5 cc. of a solution of dry HCl (5 % by weight) in absolute alcohol are added to the suspension which is stirred for about five minutes. About 30 cc. of dry ether are then added and the hydrochloride settles rapidly as a white amorphous powder. After centrifuging, the clear liquid is poured off and the precipitate is washed twice with dry ether. It is then transferred in the tube to a vacuum desiccator containing sulphuric acid and the residual ether is removed by cautious evacuation. The hydrochloride, an almost white, non-hygroscopic powder, is obtained in a yield of about 75–80 % of the picrate.

In a typical preparation 4·64 g. of "crude insulin" were taken. A rabbit unit of this material was 15 mg. 0·3281 g. picrate was obtained, of which 1 mg. was a rabbit unit. This yielded 0·2461 g. of hydrochloride which on first testing caused convulsions in a rabbit in a dose of 0·5 mg. On retesting, this dose just failed to produce convulsions although the blood sugar approached the convulsant level, while a rabbit receiving 1 mg. had severe convulsions. The total number of rabbit units in the "crude insulin" was 309. The assay of the picrate indicated 328 "units," while if 0·75 mg. be taken as the "rabbit unit" of the hydrochloride the yield from the picrate was quantitative.

Elimination of Depressor Constituent of "Crude Insulin" by means of the Picrate Purification.

In this experiment a portion of a batch of "crude insulin" which had a rabbit unit of 10 mg., was converted into the hydrochloride of which 0·75–1·0 mg. proved to be a rabbit unit.

A cat, with vagi intact, under ether anaesthesia, was used to determine the effect of the "crude insulin" and the hydrochloride on the blood pressure.

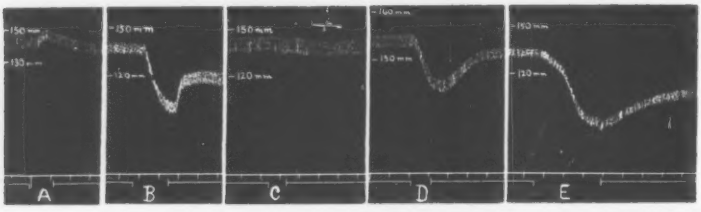


Fig. 1.

The following injections into the femoral vein were given

A. At 4.0 p.m.: 2 mg. hydrochloride.
B. „ 4.2 „ 20 „ "crude insulin."
C. „ 4.6 „ 5 „ hydrochloride.
D. „ 4.8 „ 20 „ "crude insulin."
E. „ 4.13 „ 50 „ "crude insulin."

At *A*, *B* and *D*, therefore, two rabbit units of insulin were given and at *C* and *E* five rabbit units.

The hydrochloride, then, under these conditions, had no effect on the blood pressure, while corresponding doses of "crude insulin" produced marked falls in blood pressure.

The "Isoelectric Point" Precipitate.

0·1045 g. of "insulin hydrochloride" was dissolved in 4 cc. of water in a centrifuge tube. 0·4 cc. *N* Na_2HPO_4 was added, drop by drop. It was found that after this amount had been added no further precipitate was formed. The p_H of the solution was 5·7. The precipitate was formed very promptly; standing in the cold room overnight produced no more. It was centrifuged off, washed with absolute alcohol and finally with dry ether. It was then dried by placing it in a vacuum desiccator over H_2SO_4 and evacuating cautiously. The dry precipitate, a white powder, weighed 0·0353 g.

It was dissolved in *N*/15 Na_2HPO_4 solution for testing on rabbits. The rabbit unit of the hydrochloride was 0·5 mg. and that of the precipitate 0·33 mg., so that 50 % of the activity had been removed from the solution and the precipitate was about $1\frac{1}{2}$ times as active as the hydrochloride from which it had been prepared.

This ratio was checked by testing the residual activity of the solution from which the precipitate was obtained.

The Precipitate formed by the Addition of strong Hydrochloride Acid to a Solution of "Insulin Hydrochloride."

0·13 g. of hydrochloride was dissolved in 10 cc. water. 10 % hydrochloric acid was added gradually until no further precipitate was formed. This required 5 cc. of the acid. After standing in the cold room overnight the precipitate was centrifuged down, washed with a little 3·3 % HCl, and dried in a vaccum desiccator. The weight of the precipitate was 0·02 g.

It was dissolved in water for the rabbit test. It was not quite completely soluble in water, a small amount of flocculent material remaining suspended in the liquid.

The mother liquor was nearly neutralised with NaOH and then treated with aqueous picric acid solution and the residual material recovered as picrate. It weighed 0·0815 g.

The rabbit unit of the hydrochloride was 0·5 mg., and that of the precipitate 0·25 mg. The precipitate was therefore twice as active as the hydrochloride from which it had been prepared, and 32 % of the total activity had been precipitated.

Stability of Insulin to Acid.

A batch of "crude insulin" of which the rabbit unit was 10 mg. was used in these experiments.

A solution of the "crude insulin" containing 10 mg. per cc. was made in $N/10$ H_2SO_4 and divided into 1 cc. portions. These were heated in test-tubes in a boiling water-bath for various periods, and then cooled, neutralised with $N/10$ Na_2CO_3, and injected subcutaneously into rabbits. The following results were obtained:

Ten minutes' heating. Rabbit weighed 1·6 kilo.:

Blood sugar, normal	0·107 %
" " 1 hour after injection	0·047
" " 2 hours " "	0·023

The animal had convulsions in 2 hours and was recovered by injecting glucose.

Thirty minutes' heating. Rabbit weighed 2·35 kilos.:

Blood sugar, normal	0·111 %
" " 1 hour after injection	0·036
" " 2 hours " "	0·029

Animal had convulsions in 2 hours and was recovered by injecting glucose.

One hour's heating. Rabbit weighed 1·1 kilo.:

Blood sugar, normal	0·090 %
" " 1 hour after injection	0·061
" " 2 hours " "	0·056
" " 3 " " "	0·065

Two hours' heating. Rabbit weighed 1·1 kilo.:

Blood sugar, normal	0·088 %
" " 1 hour after injection	0·055
" " 2 hours " "	0·072
" " 3 " " "	0·093

The last two results show that slow destruction of the active principle is occurring.

An aqueous solution of the same batch of "crude insulin" containing 10 mg. per cc. was heated to 100° for ten minutes. 1 cc. of the solution was then injected subcutaneously into a rabbit weighing 1·75 kilo.:

Blood sugar, normal		0·108 %
,, ,,	1 hour after injection	0·092
,, ,,	2 hours ,, ,,	0·069
,, ,,	3 ,, ,, ,,	0·081
,, ,,	4 ,, ,, ,,	0·090

Evidently in such a faintly acid solution—the p_H is about 6·1—insulin is quite unstable at 100°.

Stability to Alkali.

The same batch of insulin was used as in the experiments on stability to acid.

10 mg. were dissolved in 1 cc. $N/10$ NaOH and heated to 37° for $1\frac{1}{2}$ hours. The solution was then neutralised with 1 cc. $N/10$ HCl and injected into a rabbit weighing 1·5 kilo.:

Blood sugar, normal		0·101 %
,, ,,	1 hour after injection	0·105
,, ,,	2 hours ,, ,,	0·101

No trace of activity is left.

10 mg. were dissolved in 1 cc. $N/10$ Na_2CO_3 and heated to 37° for one hour. After neutralisation with 1 cc. $N/10$ HCl the solution was injected into a rabbit weighing 1·5 kilo.:

Blood sugar, normal		0·114 %
,, ,,	1 hour after injection	0·066
,, ,,	2 hours ,, ,,	0·048
,, ,,	3 ,, ,, ,,	0·035

The animal had convulsions in three hours and was recovered by the injection of glucose.

10 mg. were dissolved in 1 cc. $N/10$ Na_2CO_3 and heated in a boiling water-bath to 100° for ten minutes. After neutralisation the solution was injected into a rabbit weighing 1·5 kilo.:

Blood sugar, normal		0·113 %
,, ,,	1 hour after injection	0·107
,, ,,	2 hours ,, ,,	0·113
,, ,,	3 ,, ,, ,,	0·129

This result indicates complete destruction of the active principle.

Action of Trypsin.

0·5 g. of a commercial sample of trypsin was dissolved in 10 cc. $N/10$ Na_2CO_3 and 20 mg. of the same "crude insulin" which was used in the previous experiments on the stability to acid and alkali was dissolved in 2 cc. water.

The insulin solution was divided into two equal portions of 1 cc. each and to one of them was added 1 cc. of the trypsin solution, and to the other 1 cc. of a portion of the trypsin solution which had previously been heated to 100° for 30 minutes. A drop of chloroform was added to each solution.

After incubation at 37° for two hours they were injected into two rabbits.

(1) Trypsin experiment. Rabbit weighed 3·5 kilos.:

Blood sugar, normal		0·092 %
,, ,,	1 hour after injection	0·104
,, ,,	2 hours ,, ,,	0·101
,, ,,	3 ,, ,, ,,	0·104

(2) Heated trypsin control. Rabbit weighed 3·25 kilos.:

Blood sugar, normal		0·101 %
,, ,,	1 hour after injection	0·059
,, ,,	2 hours ,, ,,	0·066
,, ,,	3 ,, ,, ,,	0·074

Trypsin, therefore, promptly destroys insulin.

Action of Pepsin.

1*st experiment.* 0·3 g. of a commercial pepsin preparation was dissolved in 50 cc. *N*/500 HCl. A portion of this solution was heated to 100° for 30 minutes.

20 mg. "insulin hydrochloride," of which the rabbit unit was 0·8 mg., were dissolved in 5 cc. water.

(1) To 2·5 cc. of the insulin hydrochloride solution were added 2·5 cc. of the pepsin solution.

(2) To 2·5 cc. of the insulin hydrochloride solution were added 2·5 cc. of the heated pepsin solution.

To each of the solutions were added a few drops of chloroform. They were then placed in the hot room at 37° for 16 hours.

1 cc. of (1) (*i.e.* 2 mg. insulin hydrochloride) was injected into a rabbit weighing 2·4 kilos.:

Blood sugar, normal		0·123 %
,, ,,	1 hour after injection	0·129
,, ,,	2 hours ,, ,,	0·124

1 cc. of (2) (*i.e.* 2 mg. insulin hydrochloride) was injected into a rabbit weighing 2·3 kilos.:

Blood sugar, normal		0·109 %
,, ,,	1 hour after injection	0·077
,, ,,	2 hours ,, ,,	0·041

The animal had convulsions 2½ hours after the injection and was recovered by injecting glucose.

The experiment shows a complete destruction of insulin by pepsin.

2*nd experiment.* It was desired, if possible, to demonstrate the action of pepsin in a shorter time than in the previous experiment.

A 1 % solution of commercial pepsin of a different source from that employed in the first experiment was made in *N*/10 HCl. A portion of this solution was heated to 100° for 30 minutes. A solution of the same specimen of "insulin hydrochloride" containing 4 mg. per cc. was prepared.

(1) To 3 cc. of the insulin hydrochloride were added 3 cc. of the pepsin solution.

(2) To 2 cc. of the insulin hydrochloride were added 2 cc. of the heated pepsin solution.

A few drops of chloroform were added to the solutions, which were then placed in the hot room at 37°.

After one hour 1 cc. of solution (1) was removed, neutralised with Na_2CO_3 and injected into a rabbit weighing 2·2 kilos. After two hours a similar sample was withdrawn and injected into a rabbit. As the destruction of the insulin was found to be complete at the end of the first hour only the blood sugar record of the first rabbit is given:

Blood sugar, normal		0·107 %
,, ,,	1 hour after injection	0·113
,, ,,	2 hours ,, ,,	0·114
,, ,,	3 ,, ,, ,,	0·126

After four hours a 1 cc. sample was withdrawn from the control experiment (2) and, after neutralisation, injected into a rabbit weighing 1·9 kilo.:

Blood sugar, normal		0·120 %
,, ,,	1 hour after injection	0·074
,, ,,	2 hours ,, ,,	0·063

The animal was then given an injection of glucose and the experiment discontinued.

The insulin was completely destroyed by pepsin in one hour.

Berkefeld Filtration of "Crude Insulin."

1·04 g. of crude insulin was dissolved in 100 cc. water. The p_H of this solution was 6·1.

It was then sucked through a kieselguhr candle, except for a small quantity (6·5 cc.) which was held back for tests. Owing to traces of alkali in the candle the p_H of the filtered solution had risen to 6·6. The filter was washed by drawing 50 cc. $N/10$ Na_2HPO_4 through it.

1 cc. of the unfiltered solution was injected into a rabbit weighing 1·6 kilo.:

Blood sugar, normal		0·111 %
,, ,,	1 hour after injection	0·046
,, ,,	2 hours ,, ,,	0·030

The animal had convulsions 1½ hours after the injection and was recovered by injection of glucose after the 2nd hour blood sample had been taken.

1 cc. of the filtered solution was injected into a rabbit weighing 2·3 kilos:

Blood sugar, normal		0·115 %
,, ,,	1 hour after injection	0·085
,, ,,	2 hours ,, ,,	0·071
,, ,,	3 ,, ,, ,,	0·087
,, ,,	4 ,, ,, ,,	0·102

0·5 cc. of the sodium phosphate solution was injected into a rabbit weighing 2·5 kilos:

Blood sugar, normal		0·106 %
,, ,,	1 hour after injection	0·041
,, ,,	2 hours ,, ,,	0·031
,, ,,	3 ,, ,, ,,	0·032

The animal recovered from convulsions without requiring an injection of glucose.

It is apparent that nearly all of the active material was adsorbed by the kieselguhr filter, and that the adsorbed substance was recovered by passing the alkaline solution of sodium phosphate through the filter.

Berkefeld Filtration of "Crude Insulin" in Alkaline Solution.

0·547 g. of the preparation used in the previous experiment was dissolved in 54·7 cc. $N/10$ Na_2HPO_4. The p_H of this solution was 7·2.

A portion was reserved unfiltered for control tests and the rest was passed through a kieselguhr filter.

The p_H of the filtered liquid was 7·4 owing to traces of alkali in the candle. The filtrate was found to be sterile by bacteriological tests.

1 cc. of the unfiltered solution was injected into a 2·5 kilo. rabbit.

Blood sugar, normal	0·090 %
,, ,, 1 hour after injection	0·040
,, ,, 2 hours ,, ,,	0·047

The animal had convulsions 1¾ hours after the injection and was recovered by injection of glucose.

1 cc. of the filtered solution was injected into a rabbit weighing 2·1 kilos.:

Blood sugar, normal	0·116 %
,, ,, 1 hour after injection	0·052
,, ,, 2 hours ,, ,,	0·046

The animal had convulsions 1¾ hours after the injection and was recovered by injection of glucose.

All the rabbits used throughout these experiments were starved for 16–24 hours before the tests were made.

The blood sugars were estimated by the method of Shaffer and Hartmann [1920].

I am much indebted to Dr H. H. Dale for constant interest and helpful criticism throughout the course of the work and also for his kindness in performing the blood pressure experiment.

I wish to express my sincere thanks to Mr H. P. Marks for having carried out a large number of blood sugar determinations and to Mr W. W. Starling for valuable help in the preparation of "crude insulin."

Summary.

1. A method of purifying crude preparations of insulin is described. It consists in precipitating the active material with picric acid and converting the insoluble picrate into a soluble hydrochloride by means of an alcoholic solution of hydrochloric acid.

2. This hydrochloride is an almost white powder of which 0·5–1 mg. will lower the blood sugar of a 2 kilo. rabbit, which has been starved for 24 hours, to about 0·04 % and cause typical hypoglycaemic convulsions.

3. The characteristic reactions given by this substance are the biuret, Pauly and organic sulphur tests. The glyoxylic acid reaction is negative, and the Millon test is very faint and atypical. It contains no phosphorus.

4. From its solutions two precipitates may be obtained; one by the addition of acid and the other by the addition of alkali. The first is about twice and the second about one-and-a-half times as active as the original hydrochloride.

5. It is shown that both pepsin and trypsin destroy insulin.

6. Insulin is relatively stable to acid, but is decomposed with ease by alkali.

7. Insulin, in faintly acid solutions, is very readily adsorbed, but it may be filtered through Berkefeld filters without appreciable loss if the solution is first made weakly alkaline.

8. Insulin appears to be a very complex protein derivative.

REFERENCES.

Banting and Best (1922). *J. Lab. Clin. Med.* **7**, 464.
Best and Macleod (1923). *J. Biol. Chem.* **55**; *Proc. Amer. Soc. Biol. Chemists*, xxix.
Collip (1923). *J. Biol. Chem.* **55**; *Proc. Amer. Soc. Biol. Chemists*, xl.
Doisy, Somogyi and Shaffer (1923). *J. Biol. Chem.* **55**; *Proc. Amer. Soc. Biol. Chemists*, xxxi.
Hopkins (1921). *Biochem. J.* **15**, 288.
McCormick, Macleod, Noble and O'Brien (1923). *J. Physiol.* **57**, 234.
Shaffer and Hartmann (1920). *J. Biol. Chem.* **45**, 365.
Winter and Smith (1923). *J. Physiol.* **57**, *Proc.* xl, Feb. 17th.

XLIII. THE ESTIMATION OF AMMONIA AND UREA IN URINE AND OTHER FLUIDS.

By SAMUEL LEVY-SIMPSON and DENIS CHARLES CARROLL.

From the Biochemical Laboratories, Cambridge.

(*Received March 15th, 1923.*)

F. W. Foreman [1920] has used steam alcoholic distillation to separate volatile bases from amino acids, estimations being afterwards made by his well-known method of titrating in alcohol. He has since applied the method to mixtures of urea and ammonia, and finds that it is applicable to urine[1].

The fact that when a mixture of urea and ammonia is steam distilled in alcohol only the ammonia distils over during a certain stage of the distillation has been made use of in the Cambridge Biochemical Laboratory—at Mr Foreman's suggestion—to estimate ammonia and urea in solutions containing either or both. The method is so very rapid and easy to handle that it should be generally employed. Its use can only be attended with confidence however if the conditions for the distillation are carefully defined. It is the purpose of this paper to show that with due attention to conditions it can be made absolutely reliable. When it is borne in mind that a single determination occupies only a few minutes, the advantages of the method over the aeration and other methods at present in use will be sufficiently obvious.

Method.

The apparatus consists of a steam boiler, a large (1 litre) distilling flask and a condenser. The distilling flask, which is placed at 45° to the horizontal, is fitted with a rubber cork carrying a tap funnel containing an alcoholic solution of caustic potash coloured with phenolphthalein; and a glass tube which is bent so that it dips right down into the lowest part of the flask. This tube is connected by a movable piece of rubber tubing to the steam boiler. The side tube of the flask is connected to the condenser, which is arranged as nearly vertical in position as possible. A collecting flask containing standard HCl is placed so as to receive the distillate. A sample (5 to 10 cc.) of the fluid under investigation is taken and mixed with (90 cc. of) alcohol and placed in the distilling flask. The boiler is heated but it is not connected to the distilling flask until a vigorous jet of steam is issuing from the connecting tube. The coloured alcoholic potash solution is next run in from the tap funnel until the fluid in the distilling flask is just alkaline to phenolphthalein. If necessary more potash may be added in this way during

[1] Private communication.

the distillation. This method of rendering the fluid alkaline in order that ammonia may be distilled off has been found necessary in order to ensure that no leakage of ammonia occurs. The fluid soon boils and ammonia and other volatile bases are distilled over into the collecting flask, where they neutralise the acid placed there; and are subsequently estimated by titration with CO_2-free soda, using alizarin-red as an indicator. After a few minutes a foam or froth appears on the fluid in the distilling flask. This indicates that the alcohol has nearly all distilled off from the fluid; frothing commences when the alcohol concentration is still as high as 3 %. At the first sign of frothing, therefore, the rubber stopper of the flask is turned round so that the bent end of the steam delivery tube is turned up out of the fluid. The steam then passes through the distilling flask and condenser and carries away any ammonia present there. This "washing" is allowed to continue for $1\frac{1}{2}$ minutes and the collecting flask is then removed and titrated as described above.

Practical Notes.

1. Frothing should not be confused with the splashing and bubbling that occurs in the first few minutes. It is a steady foam that gradually spreads over the surface of the fluid. Frothing may be made very obvious by the addition of a minute amount of fat or fatty acid, but it is easily detected after a few attempts at using the apparatus.

2. The condenser tube should be arranged nearly vertically to ensure complete drainage. Otherwise the condenser must be washed through with distilled water at the end of the distillation.

3. To avoid fluid in the distilling flask being splashed over, use a large flask (about one litre) and incline it as much as possible.

Note on corrections applied to the results.

The alcohol used was 96 %; wherever alcohol content is expressed as a percentage, it is this percentage of 96 % alcohol to which we refer. For example "80 % alcohol" should read "80 cc. of 96 % alcohol in 100 cc. of fluid."

The distilled water used was not neutral to the indicator (alizarin-red) used. The correction was applied where necessary:

25 cc. distilled water found equivalent to 0·10 cc. of $N/50$ HCl.

The blank correction for the apparatus was obtained by distilling a mixture of alcohol and water until frothing:

Blank correction = + 0·2 cc. $N/50$ HCl.

The urea used was twice recrystallised. A small amount of ammonium carbonate, etc. was however still present as impurity. Thus:

4 cc. of 25 % urea yielded ammonia equivalent to 1·15 cc. $N/50$ HCl.

The correct value of the ammonium carbonate solutions was obtained by distillation in aqueous solution and by the Van Slyke modification of Folin's aeration method.

The value of ammonia is expressed throughout in terms of its equivalent standard acid.

The amount of ammonia given off before frothing depends upon the time of frothing and the rate of boiling, but these are determined by the composition and bulk of the fluid used. Our first experiments, therefore, were intended to show the relationship between percentage of alcohol, bulk of alcohol and aqueous solution, and the amount of ammonia given off before frothing.

Exp. 1. Effect of varying the percentage of alcohol with constant bulk of fluid (100 cc.) and constant volume of ammonium carbonate solution (5 cc.).

	(1)			(2)	
Percentage alcohol	Ammonia present cc. $N/50$ HCl	Ammonia distilled cc. $N/50$ HCl	Time to frothing m. s.	Ammonia distilled cc. $N/50$ HCl	Time to frothing m. s.
10	21·30	8·20	2 10	7·40	2 5
30	21·30	15·40	3 0	13·15	3 0
50	21·30	18·80	3 30	18·00	3 30
70	21·30	21·00	4 50	21·10	4 40
80	21·30	21·25	5 0	21·30	5 0
90	21·30	21·30	5 30	21·30	5 40
95	21·30	21·30	5 40	21·30	5 40

This experiment shows that only a proportion of 21·3 cc. of ammonia ($N/50$ acid) is distilled over before frothing if the percentage of alcohol is 70 or under. It is also evident that with constant total volume of fluid, increasing the percentage of alcohol increases the time up to frothing.

Exp. 2. The effect of varying the volume of aqueous solution, with constant volume of alcohol (95 cc.) and constant volume of ammonium carbonate solution (5 cc.).

Volume alcohol cc.	Volume of aqueous solution cc.	Ammonia present cc. $N/50$ acid	Ammonia distilled cc. $N/50$ acid	Calculated percentage alcohol
95	5	127·0	127·00	95
95	25	127·0	126·60	80
95	40	127·0	126·15	70
95	55	127·0	126·00	63
95	70	127·0	124·40	58
95	95	127·0	121·10	50

This experiment shows that although the large volume of 95 cc. alcohol is present, it is also necessary to have the percentage alcohol above a critical value to ensure that all the ammonia has distilled over before frothing.

Exp. 3. Effect of varying the total volume of fluid, percentage of alcohol being kept constant.

Percentage alcohol	Total volume of solution cc.	Ammonia present cc. $N/10$ acid	Ammonia distilled cc. $N/10$ acid	Time to frothing m. s.
90	100	42·60	42·65	5 20
90	50	42·60	41·50	4 0
		cc. $N/50$ acid	cc. $N/50$ acid	
90	100	42·35	42·15	5 20
90	50	42·35	42·30	4 0

This experiment shows that in addition to critical percentage, a critical volume is necessary to ensure that all the ammonia distils over before frothing. This critical volume varies with the amount of ammonia originally present in solution.

The first three experiments indicated that 90 % alcohol and 100 cc. total volume were values which ensured complete distillation of these quantities of ammonia before frothing; and our next step was to ascertain the range of ammonia concentration for which they held.

Exp. 4. Effect of varying the amount of ammonia, with constant percentage of alcohol (90 %) and constant total volume of fluid (100 cc.).

Ammonia present cc. *N*/10 acid	Ammonia distilled cc. *N*/10 acid	Ammonia distilled cc. *N*/10 acid
144·10	144·10	144·15
43·50	43·45	43·50
26·10	26·10	26·10
8·70	8·70	8·70
cc. *N*/50 acid	cc. *N*/50 acid	cc. *N*/50 acid
0·395	0·395	0·390

This experiment shows that with 90 % alcohol and 100 cc. total volume of fluid, any value of ammonia between 144·1 cc. (*N*/10 acid) and 0·395 cc. (*N*/50 acid) can be completely distilled over before frothing. We did not attempt to estimate still smaller quantities of ammonia, since the blank correction was greater than 50 % of this ammonia value. The highest value of ammonia estimated represents 5 cc. of saturated ammonium carbonate solution, *i.e.* the highest concentration possible. The values of 90 % alcohol and 100 cc. total fluid are therefore safe and accurate for this large range of ammonia values.

We then investigated whether the presence of urea in the solution would influence the proportion of ammonia given off before frothing.

Exp. 5. Effect of varying the percentage of alcohol, total volume of fluid (100 cc.) and the ammonia content being kept constant. The solution contains 4 cc. of 25 % urea.

Percentage alcohol	Ammonia present cc. *N*/10 acid	Ammonia distilled, cc. *N*/10 acid (1)	Ammonia distilled, cc. *N*/10 acid (2)
90	144·10	144·10	144·10
80	144·10	144·05	144·05
70	144·10	143·40	143·50
60	144·10	141·60	141·65

The experiment shows that the presence of urea in solution does not apparently influence the distillation of the ammonia. With 90 % alcohol and total volume of 100 cc. the whole of the ammonia distils over before frothing (cf. Exp. 1).

Exp. 6. Effect of extreme ratios of urea and ammonia, with percentage of alcohol constant (90 %) and total volume of fluid constant (100 cc.).

Saturated urea solution	0·04 % urea solution	Ammonia present cc. *N*/50 acid	Ammonia present cc. *N*/10 acid	Ammonia distilled, cc. (1)	Ammonia distilled, cc. (2)
5	—	0·395	—	0·395	0·400
5	—	—	144·10	144·05	144·15
—	5	0·395	—	0·390	0·395
—	5	—	144·10	144·20	144·10

This experiment shows that very small and very large amounts of ammonia can be estimated accurately in the presence of very large and very small concentrations of urea.

Time course of the Distillation.

It was necessary to ascertain for different percentages of alcohol the exact time relation of frothing to the end of ammonia distillation and the commencement of urea decomposition. This was done by finding the volume of ammonia given off at different periods after the commencement of distillation. The collecting flasks containing standard acid were changed at noted times. Volumes of ammonia (equivalents in $N/50$ acid) were plotted along the ordinates, and time along the abscissae, and the time curves thus obtained. The total volume of fluid in all cases was 100 cc.

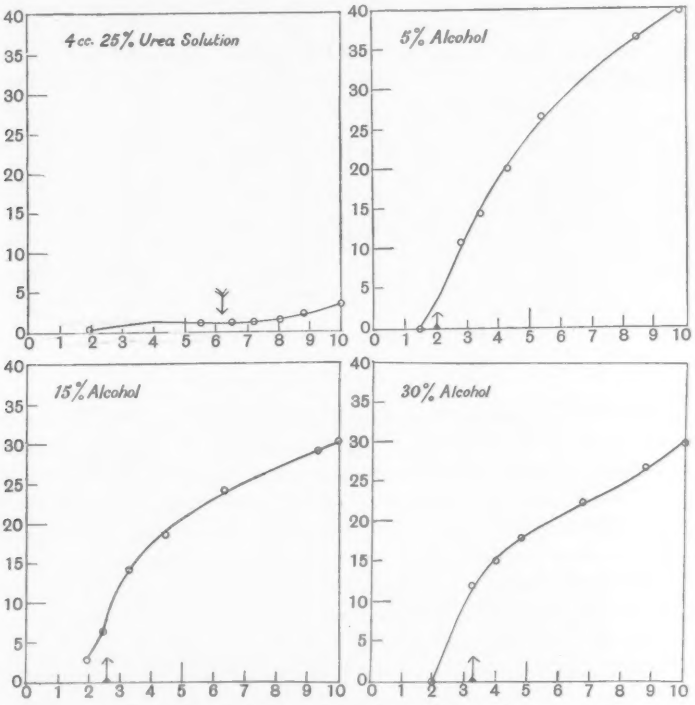


Fig. 1. Curves showing time relations of the analysis.

Abscissae = time from commencement of analysis in minutes.

Ordinates = cc. $N/50$ HCl neutralised by the ammonia distilled over.

Total bulk of fluid 100 cc. Ammonia content the same in all the curves except the first. See Exp. 6.

Arrows indicate commencement of frothing.

Practical Notes.

1. The ammonia in the condenser at the time of removing a receiving flask is inevitably collected in the next flask.

2. The slight loss of ammonia that may occur on changing collecting flasks does not affect the well-marked nature of the curves.

3. The time curve for urea alone shows that the ammonia from the impurity of ammonium carbonate is distilled over completely before frothing. The value of the ammonia impurity was confirmed by Van Slyke's method, thus permitting a correction for the remaining time curves.

Exp. 7. *Data for time curves.* The following tables illustrate the nature of the data upon which the curves are based.

The time of frothing and the value of ammonia distilled over by that time are denoted by black figures. In each case total volume of fluid is 100 cc.; urea content is constant (4 cc. of 25 % solution) and ammonia content is constant (21·40 cc. *N*/50).

(1) Urea alone in 100 cc. 90 % alcohol

Time m. s.	Ammonia distilled cc. *N*/50
2 0	0·1
4 0	0·45
6 30	**1·10**
7 10	1·10
8 0	1·50
8 50	2·10
10 50	5·30
13 0	10·10
5 20	15·30

(2) Urea and ammonia in 30 % alcohol

Time m. s.	Ammonia distilled cc. *N*/50
2 0	0·3
3 20	**12·1**
4 0	15·2
4 50	18·1
6 50	22·5
8 50	26·7
11 50	33·3
15 0	40·0

(3) Urea and ammonia in 90 % alcohol

Time m. s.	Ammonia distilled cc. *N*/50
2 0	5·50
4 0	18·90
5 30	**21·40**
6 10	21·40
6 50	21·45
7 40	21·85
9 40	24·35
11 40	27·55
15 0	32·85

N.B. 1·10 cc. represents impurity (*vide supra*).

Deductions from Time Curves.

The curves for percentages of alcohol of 70 and under (Figs. 1 and 2) show that there is no appreciable time interval between the end of ammonia distillation and the commencement of urea decomposition. The ammonia from the urea decomposition distils over before the whole of the ammonia of the ammonium carbonate has been distilled. With these low percentages it is therefore impossible to stop distillation at any definite time and obtain a correct result. This explains the discrepant results obtained through not using a sufficiently high percentage of alcohol. Frothing in these cases is of no value as a time mark. The 80 % alcohol curve shows a minimum period of zero distillation but it is not absolutely flat and must therefore be classified as unreliable for accurate work. The 90 % alcohol curve, however, shows a well-marked flat zero period, which extends over an appreciable time; and during which no ammonia distils over. Frothing (indicated by a vertical arrow) is seen to fall at the commencement of this flat zero period, and marks in this case the completion of ammonia distillation. It therefore serves as a valuable signal

for the cessation of distillation. The flat zero period represents the safety interval during which no ammonia distils over, and this is followed by urea decomposition. 90 % alcohol and a total volume of 100 cc. are therefore the conditions necessary for accurate ammonia distillations in the presence of urea.

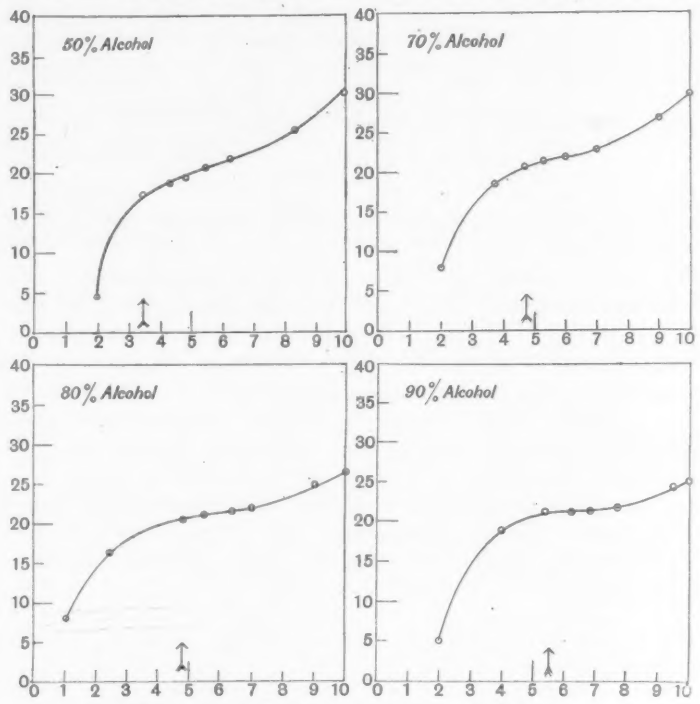


Fig. 2. Curves showing time relations of the analysis.
Abscissae = time from commencement of analysis in minutes.
Ordinates = cc. $N/50$ HCl neutralised by the ammonia distilled over.
Arrows indicate commencement of frothing.
The same bulk of fluid was used for each curve. The ammonia content was the same for each curve.

If working with unknown solutions a time curve for the conditions of experiment will show immediately whether the results obtained are reliable. If the curve resembles that for 90 % alcohol, the conditions are correct. If the zero period is absent or very short it is due to too low a percentage of alcohol or too small a total volume of fluid. This can be remedied accordingly. Thus the great value of an initial time curve for any accurate work is evident.

The Estimation of Urea.

This is done by converting the urea into ammonium carbonate by the action of a strong solution of urease. At 40° C. complete conversion was

invariably obtained in 20 minutes. The urease solution was buffered to p_H 7·02 by means of a solution consisting of suitable proportions of 3 molar caustic potash and molar acid potassium phosphate solutions.

The effect of such a buffered urease solution on the analysis was first investigated.

Exp. 8. Analysis of 100 cc. fluid containing 1 cc. ammonia and 9 cc. strong urease solution buffered to p_H 7·02.

Cc. alcohol	Ammonia present cc. $N/50$	Ammonia found cc. $N/50$
90	44·10	44·15
90	44·10	44·10
90	44·10	44·10

This experiment shows that urease has no effect upon the amount of ammonia distilled over up to frothing from 100 cc. of a 90 % alcohol solution of ammonia.

Solutions containing 25 % and 0·04 % of urea were next analysed and the results compared with those given by the aeration method.

Exp. 9. Analysis of 1 cc. of 25 % urea solution.

Correct result 80·10 cc. $N/10$. Results in cc. $N/10$.

Method	1st exp.	2nd exp.	3rd exp.	Mean
Steam-alcohol distillation	80·05	80·10	80·10	80·08
Van Slyke aeration	80·10	80·15	80·15	80·13

Exp. 10. Analysis of 10 cc. of 0·04 % urea solution in 100 cc. 90 % alcohol.

Results in cc. $N/50$.

Method	1st exp.	2nd exp.	3rd exp.	Mean
Steam-alcohol distillation	6·35	6·37	6·37	6·37
Van Slyke aeration	6·34	6·33	6·36	6·34

The method is therefore quite accurate for the estimation of urea in either strong or weak solutions.

Application to Urine.

We then successfully attempted to apply the method to the analysis of urine.

In accordance with the recommendations made earlier in this paper our first experiment was to obtain a "time-ammonia" curve for the apparatus when 5 cc. normal urine, 90 cc. alcohol, and the equivalent in 5 cc. of ammonium carbonate solution of about 45 cc. of $N/50$ acid were present in the distilling flask. The curve obtained is shown in Fig. 3 c. The actual values are given below, Exp. 16.

The curve is of the type given by the 90 % alcohol solutions shown in Fig. 2. All the ammonia has therefore been distilled over before frothing commences. The conditions were therefore assumed to be correct and the following experiments were made.

A series of analyses of normal urine were made by this method and compared with the results given by the Van Slyke modification of the Folin aeration method.

Exp. 11. Analysis of 5 cc. urine in 100 cc. fluid containing 90 % alcohol.

Results in cc. *N*/50.

Method	1st exp.	2nd exp.	3rd exp.	4th exp.	Mean
Steam-alcohol distillation	7·25	7·20	7·24	7·25	7·235
Van Slyke aeration	7·22	7·24	7·25	7·25	7·240

Exp. 12. Analysis of 5 cc. urine in 100 cc. 90 % alcohol containing added ammonium carbonate.

Results in cc. *N*/10.

Method	1st exp.	2nd exp.	3rd exp.	Mean
Steam-alcohol distillation	44·12	44·10	44·15	44·120
Van Slyke aeration	44·10	44·15	44·10	44·120

This is as large a *quantity* of ammonia as is ever present in the quantities of urine which we recommend using, either before or after the urea has been converted into ammonium carbonate. The time curve (not shown) of this experiment was quite satisfactory.

These experiments show that the method is quite accurate for the estimation of ammonia in normal urine.

The urea was next estimated. 1 cc. of urine was acted on by 9 cc. of standard urease solution at 40° for 20 minutes. All the urea was found to be converted into ammonium carbonate by this procedure.

Exp. 13. Analysis of 1 cc. urine after treatment with urease to convert the urea into ammonium carbonate. 100 cc. of 90 % alcohol were used.

Results in cc. *N*/50.

Method	1st exp.	2nd exp.	3rd exp.	4th exp.	Mean
Steam-alcohol distillation	35·40	35·45	35·45	35·45	35·44
Van Slyke aeration	35·40	35·45	35·45	35·45	35·44

Again there is complete agreement with the Van Slyke-Folin method.

The method is therefore unaffected by urease in the presence of urine and it is accurate for the estimation of ammonia and urea in normal urine.

We then ascertained whether the method was equally accurate for the estimation of ammonia and urea in pathological urines. To do this we analysed samples of urine containing various foreign substances both with and without urease.

Exp. 14. Analysis of 5 cc. urine in 100 cc. 90 % alcohol in the presence of foreign substances.

Results in cc. *N*/50.

Substance introduced	Ammonia present	Ammonia found	Substance introduced	Ammonia present	Ammonia found
1 g. glucose	7·00	7·00	1 cc. butyric acid	7·00	6·95
,,	7·00	7·00	1 cc. conc. butyric acid	7·00	7·00
1 g. serum albumin	7·00	6·95	1 cc. 4 % aceto-acetic acid	7·00	7·00
,,	7·00	6·95	,, ,,	7·00	7·00
1 cc. conc. acetone	7·00	6·98			
,,	7·00	6·95			

The results found in the presence of urease were exactly similar.

We conclude from these experiments that the method is applicable with great accuracy to the estimation of ammonia and urea in all urines whether normal or pathological.

Although the alcohol used may be easily recovered from the distillate after titration it is desirable that as little as possible be used when a large number of estimations are being made. We therefore investigated the effect of halving the volume of alcohol used, so that the total volume of fluid was only 50 cc. instead of 100 cc. We first repeated Exp. 11 using this smaller bulk.

Exp. 15. Analysis of urine in 50 cc. of fluid.

Cc. alcohol	Cc. urine	Ammonia present cc. $N/50$	Ammonia found cc. $N/50$
45	5	42·35	42·30
45	5	42·35	42·35
40	1+9 cc. urease	39·12	39·15
40	1+9 cc. „	39·12	39·10

For these values therefore the correct result is obtained with the smaller bulk.

From Exp. 3 it will be remembered, however, that 42·6 cc. $N/10$ ammonia (the maximum amount which can possibly be present in our urine estimations) is not quite all distilled over before frothing commences when a total bulk of 50 cc. of 90 % alcohol is used. The same holds good for urine and is an objection to the use of a smaller quantity of alcohol than 90 cc.

The time curves were then investigated and compared with those of equal percentage alcohol content but of double the volume of fluid.

The curves are shown in Fig. 3.

Exp. 16. *Time curves for urine.* See Fig. 3. The following table illustrates the nature of the data from which the curves were constructed. Frothing occurred at the time denoted by black figures.

Analysis of 5 cc. urine A in 100 cc. and B in 50 cc. 90 % alcohol. Ammonia content = 42·35 cc. $N/50$.

Time from commencement		Cc. $N/50$ ammonia distilled over	
A	B	A	B
m. s.	m. s.		
2 0	2 0	16·0	18·2
5 0	4 20	42·0	41·7
5 20	**4 50**	**42·3**	**42·3**
6 0	5 20	42·3	42·3
6 40	6 0	42·3	42·8
7 30	8 0	42·4	43·3
9 0	10 0	43·1	44·6
12 0	13 30	45·1	46·6
15 0	—	49·7	—

From this experiment it is clear that in the smaller bulk the zero period is smaller than in the larger bulk of equal alcohol percentage; and, bearing this in mind, there are two objections to using the smaller bulk.

(1) The zero period is shorter in the smaller bulk than in the larger bulk of equal alcohol concentration and therefore if the distillation is not stopped exactly at the commencement of frothing an error may be introduced.

(2) If the ammonia present is greater than 42·35 cc. $N/50$ all the ammonia may not have distilled over before frothing commences.

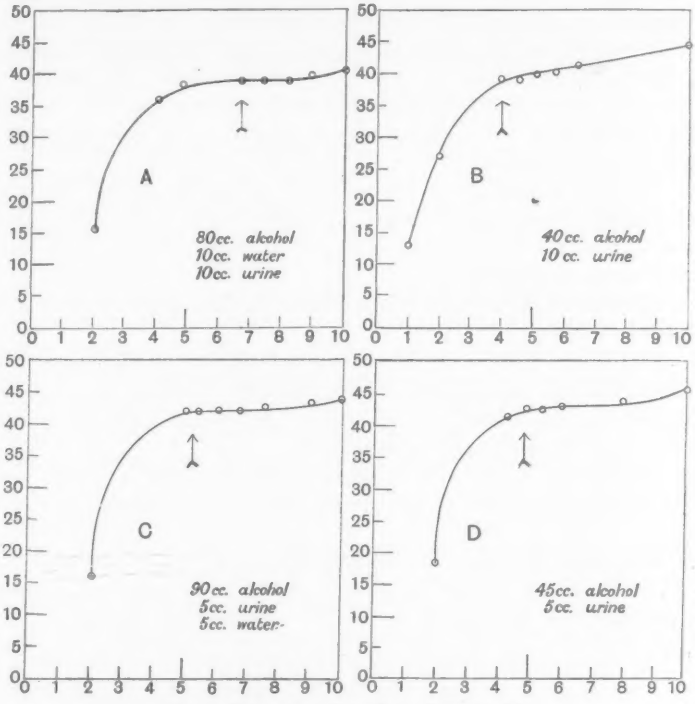


Fig. 3. Time curves for urines. See Exp. 16.
Ordinates = cc. $N/50$ HCl neutralised by the ammonia distilled over.
Abscissae = time in minutes from commencement of analysis.
Arrows indicate commencement of frothing.
The ammonia content was the same in all cases.

This error may be avoided, of course, by using smaller quantities of urine.

For general work therefore we recommend conducting the analysis in 100 cc. of 90 % alcohol which we have shown to give accurate results in all cases. For analysing *quantities* of ammonia less than 42·35 cc. $N/50$, however, a total volume of 50 cc. may be used with perfect accuracy.

Summary and Conclusion.

1. It is shown that the estimation of ammonia and urea by means of a steam-alcohol distillation is accurate to a high degree for all quantities of ammonia investigated, from 0·395 cc. $N/50$ to 144·1 cc. $N/10$ ammonium carbonate, which represents the analysis of a saturated solution of this salt. The small differences shown in some results are within the limits of error of the titration.

2. The conditions governing the steam alcohol distillation of ammonia were thoroughly investigated and it was found that when the analysis was conducted in 100 cc. of 90 % alcohol all the ammonia was distilled over before frothing commenced. This gave accurate results for all concentrations of ammonia up to saturation.

3. It is shown that the accuracy of the method in any particular case may be tested by obtaining a time analysis curve, the form of which indicates whether correct results are being obtained; although the ammonia content may not be otherwise known.

4. The method is proved to be accurate for the investigation of the action of urease on urea.

5. The method is rapid. A complete determination of ammonia and urea may be made with one apparatus in 30 minutes, including the time taken for the conversion of urea into ammonium carbonate. A determination of ammonia requires seven minutes only.

6. The method is applied successfully to the estimation of ammonia and urea in urines and is shown to hold good for pathological as well as for normal urines.

In our opinion the accuracy, rapidity, and facility of manipulation which the method possesses render it more desirable as a means of estimating ammonia and urea than any of the methods at present in use.

REFERENCE.

Foreman (1920). *Biochem. J.* **14**, 451.

XLIV. NOTE ON THE ESTIMATION OF UREA BY UREASE.

By GEORGE MACFEAT WISHART.

From the Institute of Physiology, University of Glasgow.

(*Received April 24th, 1923.*)

In the course of an investigation into the reason for a series of erroneous results obtained by the Soja bean-urease method for the estimation of urea, it was discovered that the estimation could be carried out much more rapidly and easily by doing the hydrolysis of the urea and the evacuation of the formed ammonia simultaneously. Curiously enough, if the bean be used in its crude powdered state, it is possible to add to the urea-containing solution the requisite quantity of sodium carbonate to liberate the formed ammonia without interfering with the hydrolytic action of the urease; the alkali would appear to be taken up by some constituent of the bean, possibly the proteins, and thus prevented from exerting its inhibitory action on the enzyme.

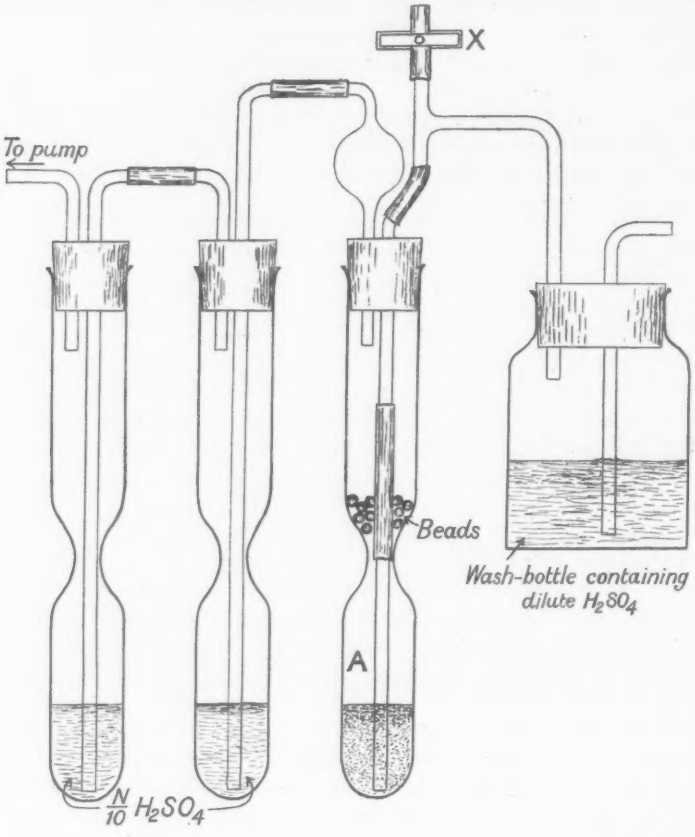
A large number of experiments were performed in which the vessel containing the $N/10$ H_2SO_4 for absorbing the liberated ammonia was connected with a burette, and thus the course of the evolution of the ammonia could be followed, minute by minute. Such experiments served for a comparison of various types of air-inlet tubes, and for the determination of the optimum temperature, and the optimum quantities of the reagents. In the course of these experiments it was found that the more rapid the aeration, the more rapidly could the estimation be completed; *e.g.* in one experiment with an air-current of 6 litres per minute, 75 % of the urea-ammonia had been liberated in 10 minutes, while with an air-current of 10 litres per minute, 92·4 % of the theoretical amount was liberated within the same time.

To provide for as rapid an air-current as possible, and at the same time to prevent loss of ammonia, and frothing of the solutions, the apparatus was modified as shown in the diagram.

Tube *A* and the absorbers are made from the tubes of an ordinary ammonia absorption apparatus. The clip at *X* allows of the saturated sodium carbonate solution being introduced after the whole apparatus is connected up and the air-current started, without any disjointing of the tubing, thus making loss of ammonia impossible.

The constrictions in all three tubes should be placed at such a level that the capacity of the lower bulb is about three times the volume of fluid to be placed within it. This arrangement, along with the use of glass beads in tube *A*, renders the use of any anti-frothing solution unnecessary in most cases.

To complete the estimation in the times given, the pump should be capable of drawing air through the apparatus at a rate of not less than 5½ litres per minute. The water-bath temperature should be maintained at about 40°.



Method of Estimation.

In tube *A* place 20 cc. water, 5 cc. urine, and 3–5 g. Soja bean meal (varying amounts are required according to the condition of the bean; a slight excess will not vitiate the result). Mix the contents of the tube and insert the central tube and glass beads as shown, immerse bulb in water-bath, then connect up to collecting tubes containing a known amount of decinormal acid. Start the pump and introduce about 2 cc. of a saturated solution of sodium carbonate from a pipette by holding the nozzle of the pipette at *X* and opening the clip. Close the clip and aerate for 40 minutes.

A smaller apparatus with tubes of about 100 cc. capacity, and using $N/50$ acid, has been found useful for estimating small amounts of urea. The quantities for this apparatus are: 5 cc. of urea-containing fluid (content not more than 10 mgm. urea); 1 g. bean meal; and 0·5 cc. saturated sodium carbonate solution. Aeration with this smaller apparatus, and using these quantities, is complete in 20 minutes.

Since the time required for complete evacuation of the ammonia depends on the velocity of the air-current and the amount of bean meal used, it is well to determine that all conditions are correct by doing one estimation on a solution containing a known amount of pure urea. The bean meal liberates a certain small amount of ammonia itself, so that, for accurate work, it is necessary to estimate this by doing a blank experiment, using water in place of the urea solution.

As examples of the results obtained by this method the following may be given.

Large apparatus ($N/50$ acid and alkali used):

Experiment	Theoretical amount	Estimated amount
1	1 %	0·9948 %
2	1	0·9936
3	1	0·9912

Small apparatus:

1	0·004 %	0·00408 %
2	0·004	0·00396
3	0·004	0·00402

Experiments on the estimation of urea in urines with and without added quantities of urea gave equally satisfactory results. In estimating urea in urine by the larger apparatus, it is usually advisable to dilute the urine so that it will contain approximately 1 % of urea.

XLV. THE ACCURACY OF THE DALE AND EVANS METHOD OF DETERMINING THE HYDROGEN ION CONCENTRATION OF BLOOD.

By HAROLD TAYLOR.

From the Physiological Laboratory, Manchester.

(*Received April 27th, 1923.*)

The method employed by Dale and Evans [1920] of finding the hydrogen ion concentration of blood is to place the blood in a small thin collodion tube and to suspend this in an isotonic saline solution. The whole is allowed to stand for about half-an-hour to allow equilibrium to be attained, and the hydrogen ion concentration of the outside solution is then determined by an indicator method. The hydrogen ion concentration of this solution is then taken as that of the blood. The hydrogen ion concentration determined by this method does not, however, according to Evans [1920] agree with the results obtained by the use of a hydrogen electrode placed in the blood, the hydrogen electrode giving, in his hands, results which were about 0·2 p_H smaller than those obtained by the dialysis method. The diffusion method is supposed to be accurate to 0·02 p_H; at any rate the p_H of the dialysate can be determined to that degree of accuracy. Since the phosphate solutions used for comparison with the dialysate are standardised with a hydrogen electrode, it is quite clear that the p_H of the dialysate must be approximately correct, and hence the difference must be due, either to the presence of the collodion membrane or to some effect of the blood on the electrode.

Evans in his paper suggests a number of possible causes to account for the difference observed, but he does not consider the fact that even in equilibrium the concentration of the hydrogen ions may not be the same on both sides of the membrane. Such would be the case if a Donnan equilibrium were set up at the membrane. Considering the proteins of blood as providing ions incapable of diffusing through the collodion membrane we have the conditions necessary for the existence of a Donnan equilibrium, *i.e.* on one side of the membrane an indiffusible ion, along with diffusible ions, and on the other side diffusible ions only. Thus when equilibrium is set up there will not be an equal distribution of diffusible ions on the two sides of the membrane. Such a state has actually been demonstrated by Loeb [1921] in the case of

gelatin solution inside a collodion tube, the concentrations of the hydrogen ions being unequal on the two sides of the membrane.

If two liquids on opposite sides of a membrane be in equilibrium and the concentrations C_1 and C_2 of the diffusible ions be not the same on both sides, then there must be a potential difference across the membrane. This potential difference has been demonstrated by Loeb [1921] and whatever be the cause of the inequality of concentration it can be shown thermodynamically [Hill, 1923] that the potential difference E must be of the form

$$E = \frac{R \cdot T}{n \cdot F} \log \frac{C_1}{C_2}.$$

[n = valency of ion; F = electrical charge per equivalent.]

Hence by measuring the potential difference between the blood inside and the saline solution outside the ratio of the concentration of hydrogen ions in the blood to that in the outer solution can be determined. In the case of hydrogen ions at 18° the formula reduces to $E = 59\ [p_{\mathrm{H}}\ (\text{inside}) - p_{\mathrm{H}}\ (\text{outside})]$ millivolts. It can be shown also that, in the case of a membrane potential, the side on which the concentration of hydrogen ions is the lower is the positive side.

In the presence of a considerable concentration of diffusible ions the difference of concentration occurring in a Donnan equilibrium must be small. In the case of blood therefore no considerable potential difference can be expected.

Method of measurement of membrane potential.

The potential difference across the membrane was measured by means of a potentiometer and two saturated KCl calomel electrodes. In many of the observations a high resistance galvanometer of extreme sensitivity was employed. The apparatus was the same as used in the actual determination of the hydrogen ion concentration by the Dale and Evans method. The small tube into which the saline was put was located in a wider tube, which was large enough to hold the whole of the apparatus when the vulcanite top had been placed on. The amount of saline in the small glass tube was arranged so that when the dialysing tube containing the blood was placed in it the saline came up to the top of the glass tube. When the whole was ready it was corked up and left for about three-quarters of an hour to ensure that equilibrium was attained. This is longer than the time generally allowed in hydrogen ion determinations. The outer tube was then filled with saturated KCl solution up to the lower rim of the vulcanite top. The cork of the dialysing tube was then removed, one saturated KCl calomel electrode placed in the blood and the other in the outer KCl solution. The calomel electrodes made contact through capillary points so as to avoid contaminating the blood with KCl. The potential difference was then measured between the two electrodes. Enough electrical contact was obtained round the edge of the vulcanite top, between

the dialysate and the KCl outside, for the readings to be made easily. The cell employed therefore was as follows:

Calomel electrode	Saturated KCl	Blood	Membrane	0·85 % saline	Saturated KCl	Calomel electrode

Due allowance was made for any difference between the calomel electrodes employed by testing them together in the same solution. The results are given in the following table.

No. of experiment ...	1	2	3	4	5	6
P.D. (millivolts) Blood positive	2·44	2·90	2·31	0	−3·45	+1·15
p_H (inside) − p_H (outside)	0·041	0·049	0·039	0	−0·058	0·020
$\frac{c_H \text{ inside}}{c_H \text{ outside}}$	0·91	0·83	0·91	1·0	1·14	0·95
No. of experiment ...	**7**	**8**	**9**	**10**	**11**	**12**
P.D. (millivolts) Blood positive	−5·3	−0·9	3·23	0·1	0·85	−0·5
p_H (inside) − p_H (outside)	−0·090	−0·015	0·055	0·002	0·018	−0·009
$\frac{c_H \text{ inside}}{c_H \text{ outside}}$	1·22	1·035	0·88	0·99	0·96	1·02
No. of experiment ...	**13**	**14**	**15**	**16**	**17**	**18**
P.D. (millivolts) Blood positive	2·2	1·3	0·76	4·42	2·54	1·66
p_H (inside) − p_H (outside)	0·037	0·022	0·013	0·075	0·043	0·028
$\frac{c_H \text{ inside}}{c_H \text{ outside}}$	0·92	0·95	0·97	0·86	0·91	0·94
No. of experiment ...	**19**	**20**	**21**	**22**		
P.D. (millivolts) Blood positive	0·45	0·66	0·53	0·75		
p_H (inside) − p_H (outside)	0·008	0·011	0·009	0·013		
$\frac{c_H \text{ inside}}{c_H \text{ outside}}$	0·98	0·97	0·97	0·97		

In the above table, in spite of small individual variations, the ratio of c_H (inside) to c_H (outside) is consistently about unity. Its average value is 0·97, the average deviation from this value being only 0·06 (about 6 %), the probable error of the mean value only about 0·01 (about 1 %). A ratio of 0·97 between the true c_H of the blood and that of the dialysate corresponds to a p_H difference of only 0·013, which is outside the limits of accuracy of measurements by the dialysis method. The average deviation in the table corresponds to a p_H difference of 0·027 which is of the same size as the admitted error of the dialysis method. Hence, for practical purposes we may regard the method of Dale and Evans as giving correctly the hydrogen ion concentration of the blood as existing at the moment when the dialysis has been completed: for greater exactness 3 % may be subtracted from the c_H or 0·013 added to the p_H, to allow for the membrane effect.

If the membranes are used several times for determinations of the hydrogen ion concentration, they become stained and in such cases potential differences up to 11 millivolts, are obtained. It was found, however, that if 0·85 % saline

was placed on both sides of these membranes a potential difference was set up across them of the same order of size as obtained with blood on one side. Hence the stained membranes, for some unknown reason, introduce a large error into the hydrogen ion determinations. The membranes therefore were always tested before use by finding the membrane potential with saline on both sides. In most cases no potential difference was obtained but a small P.D. was found when the membranes had become slightly stained while testing for leaks. Thus for accurate determinations of the hydrogen ion concentration new membranes should be employed, or at any rate such as have been used before should be carefully tested before being used again.

SUMMARY.

In the Dale-Evans method of determining the hydrogen ion concentration of blood it was conceivable that an appreciable error might occur owing to a difference of concentration (due for example to a Donnan equilibrium) produced by the membrane itself. Such an effect would result in a measurable electrical potential difference. The actual P.D. has been measured and found to be small. On the average the hydrogen ion concentration in the blood is only 3 % less than in the dialysate, an amount smaller than the error of the method. For practical purposes, therefore, the c_H of the dialysate is identical with that of the blood as existing at the moment when dialysis is complete.

I wish to express my indebtedness to Dr R. E. Conway for the provision of the Dale-Evans apparatus, and for valuable suggestions.

REFERENCES.

Dale and Evans (1920). *J. Physiol.* **54**, 167.
Evans (1920). *J. Physiol.* **54**, 353.
Hill (1923). *Proc. Roy. Soc.* A, **102**, 705.
Loeb (1921). *J. Gen. Physiol.* **3**, 667.

XLVI. THE INFLUENCE OF REACTION ON THE OXIDATION OF THE ANTISCORBUTIC FACTOR IN LEMON JUICE.

By SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute.

(*Received May 7th, 1923.*)

The earliest investigations of experimental scurvy have revealed that reaction influenced the stability of the antiscorbutic factor. Holst and Frölich [1912] were the first to draw attention to the fact that in acid solution the antiscorbutic potency displays greater stability than in alkaline solution. This observation has since been confirmed by various workers [Harden and Zilva, 1918; Hess and Unger, 1919; La Mer, 1921]. Further work on the stability of the antiscorbutic factor has shown that oxidation has also a destructive action on the potency of active solutions [Hess and Unger, 1921; Zilva, 1921; Dutcher, Harshaw and Hall, 1921].

This paper deals with the elucidation and correlation of the inactivating influences of reaction and oxidation. The experiments were instituted as a result of some suggestive information obtained in connection with another inquiry on adsorption, which is still in progress. Evidence is here produced which shows that in the case of decitrated lemon juice the destruction of the factor is a process of oxidation which proceeds rapidly in alkaline solutions and slowly in an acid medium. Under anaerobic conditions alkaline reaction seems to have no inactivating influence; in presence of air when the hydrogen ion concentration is raised the destruction of the antiscorbutic potency is delayed. An active juice made approximately $N/20$ alkaline (p_H 12·5) and kept at this hydrogen-ion concentration for 24 hours under strictly anaerobic conditions did not deteriorate to a marked extent, whilst the same juice and of the same reaction kept exposed to the air during the same period failed to protect or even to delay the onset of scurvy in guinea-pigs in a dose about 5–6 times larger than that which protected the animals from the disease when the juice was kept anaerobically. Furthermore, when the hydrogen-ion concentration was brought up to p_H 2·2 the inactivation by aeration at 100° was definitely delayed.

At p_H 12·5, in the presence of air and at room temperature, the destruction of the antiscorbutic factor proceeds fairly rapidly. After three hours it is almost completely destroyed whilst after half an hour the activity is diminished to a considerable extent.

Experimental.

All the preparations were tested out on guinea-pigs. The animals were kept on a scorbutic diet of oats and bran supplemented by a daily ration of not more than 40 cc. autoclaved milk. On this diet guinea-pigs develop scurvy in about 10–15 days and succumb to the disease after about four weeks. On the other hand when this diet is supplemented by an adequate antiscorbutic dose the animals thrive well on it.

The doses to be tested were administered daily *per os* after the animals had been on the scorbutic diet for about 14 days. In the case of a positive result the duration of the experiment was two months, after which time the guinea-pigs were chloroformed and submitted to a post mortem examination.

The Influence of Alkalinity on the Antiscorbutic Factor under Aerobic and Anaerobic Conditions.

The citric acid was removed from the lemon juice by the addition of excess of calcium carbonate and two volumes of absolute alcohol. After filtration, the solution was concentrated in a vacuum at 40–50° and eventually brought up to the original volume of the juice. The reaction was then adjusted colorimetrically to p_H 6·6–6·8. In the aerobic experiments this adjusted juice was made approximately $N/20$ by the addition of normal sodium hydroxide (1·3 cc. of N NaOH to 23·7 cc. of juice) and allowed to stand in a conical flask in the laboratory for 24 hours before being administered to the experimental animals. Great precautions were taken in the anaerobic experiments to exclude air. The adjusted decitrated juice was introduced into bottles of small capacity (about 50 cc.) immediately after the addition of the requisite alkali for the production of a $N/20$ alkaline solution, the bottle was closed with a tight-fitting rubber stopper containing a glass tube with a tap and rapidly exhausted by means of an air pump. The bottle was then placed under a bell-jar over alkaline pyrogallic acid which was in its turn exhausted. The hydrogen-ion concentration of samples of decitrated juice made alkaline was determined electrometrically both immediately after the addition of the alkali and after storage under anaerobic conditions. No significant difference could be detected in the reaction. The actual figures found in one of these cases were as follows:—immediately after the addition of alkali $p_H = 12·12$, the same juice kept under anaerobic conditions for 24 hours $p_H = 12·09$. When, however, the juice was made alkaline and allowed to stand exposed to the air the hydrogen-ion concentration gradually increased. I should like to take this opportunity of thanking Dr E. E. Atkin for having kindly carried out the electrometric estimations.

At the expiration of 24 hours the aerobic and anaerobic solutions were acidified and fed to the animals. These preparations were made daily. Doses of 1·5, 3, 5 and 7 cc. were given of each preparation. The aerobic alkaline solutions were found quite inactive, whilst the same alkaline solutions kept

anaerobically were found to be protective even in the lowest dose of 1·5 cc. Fig. 1 gives the weight curve of the animals receiving the highest dose.

It was further of interest to ascertain the time taken to bring about the total inactivation of the antiscorbutic factor in a solution of the above alkalinity when kept exposed to the air. For this purpose the decitrated lemon juice was made approximately $N/20$ alkaline as before and kept in small open conical flasks at room temperature for 0·5 hour, 1 hour and 3 hours. After the respective times of treatment, the solutions were acidified by the addition of citric acid (0·1 g. to 10 cc.) and administered to the animals the same day. The tests have disclosed that after keeping the decitrated lemon

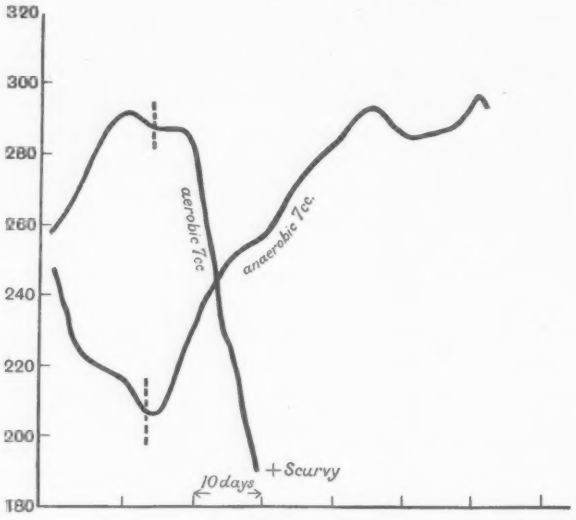


Fig. 1.

juice alkaline for one hour a daily dose of 5 cc. showed no potency. The same dose of decitrated lemon juice kept alkaline for 0·5 hour was found to be active as will be seen from Fig. 2 although the animal receiving this dose was found to show signs of incipient scurvy when chloroformed after two months. Doses of 1·5 cc. and 3 cc. of the same preparation were however ineffective to prevent or even to delay the onset of scurvy. As the minimum protective dose of decitrated lemon juice is 1–1·5 cc., it seems that about 80 % of the antiscorbutic factor has been destroyed in half an hour.

The Influence of Acidity on the Inactivation of the Antiscorbutic Factor by Aeration at 100° *C.*

In these experiments the lemon juice was prepared as in the previous experiments and adjusted to p_H 6·6–6·8. Some of the juice was brought to p_H 2·2–2·4 by the addition of citric acid. Both preparations were boiled under

a reflux condenser, air being aspirated through the boiling liquid. The decitrated lemon juice of p_H 6·6–6·8 was treated in this way for one hour. The acidified juice was aerated for one hour and for two hours.

Daily doses of 1·5, 3 and 5 cc. of the three preparations were tested out on animals. In all the three preparations 1·5 cc. of the solution failed to show any protective properties, the higher dose of 3 cc. failed to protect in the neutral preparation and in the acid solution aerated for two hours. This dose of the acid preparation which was aerated for one hour offered partial protection —the animal surviving for two months although it was in a declining and scorbutic condition at the end of that period. The difference in the degree of inactivation was best evident in the experiment in which the 5 cc. daily doses

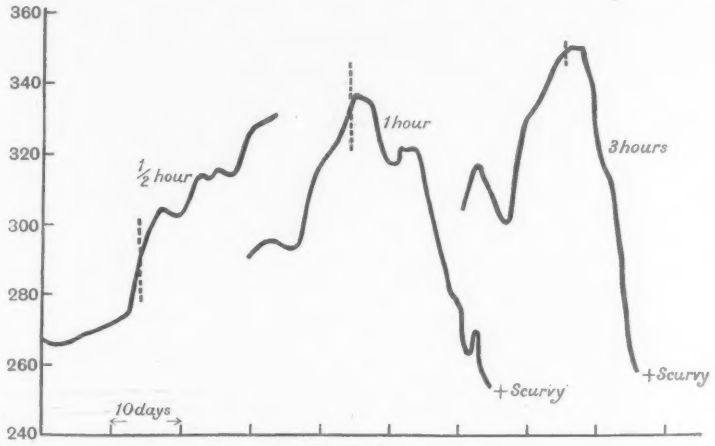


Fig. 2.

were used. Fig. 3 gives a graphic presentation of the weights of the animals which were given 5 cc. daily of each of the preparations. The animal which received the acid preparation aerated for one hour was chloroformed after two months and although some mild signs of scurvy were established it was nevertheless evident that the guinea-pig could have lived for some time longer. The animal which received the same dose of neutral preparation aerated for one hour commenced declining slowly after 15 days and died after 40 days from *acute* scurvy. Doubtful protection was achieved by 5 cc. of acid preparation aerated for two hours. These experiments show that the acidity undoubtedly had a protective action on the inactivation of the antiscorbutic factor by aeration at 100°.

Decitrated lemon juice as prepared above contains 0·8 %–1 % of solids, the best part of which consists of sugars. It is, therefore, reasonable to assume that the increase in the hydrogen-ion concentration when the alkaline juice is exposed to the air is due to the production of acids from the sugar, which according

to the extensive researches of Nef is oxidised in alkaline solution to various organic acids. Although there is a certain parallelism between the formation of acid and the inactivation of the antiscorbutic factor in the experiments described, in so far that both take place in alkaline solution in the presence but not in the absence of air, the parallelism is not complete. It is seen that about 80 % of the factor is destroyed by the treatment in about half an hour. Preliminary investigations show that the formation of acids proceeds at a much slower rate. After half an hour the hydrogen-ion concentration is only

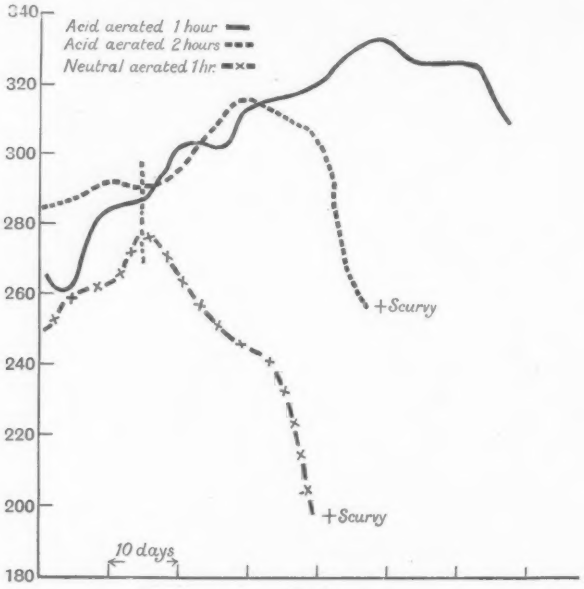


Fig. 3.

just perceptibly increased. Whether the inactivation of the antiscorbutic factor is due to an intermediate change of the sugar before the formation of the acid or whether it is a change in which the sugar is not concerned cannot be answered from the experimental evidence so far available. The writer hopes that investigations on the subject which are now in progress may conduce to the elucidation of this problem.

Summary.

Decitrated lemon juice made approximately $N/20$ alkaline ($p_{\text{H}} = 12{\cdot}5$) and kept exposed to the air at room temperature loses about 80 % of its antiscorbutic potency in half an hour. After three hours a daily dose of 5 cc. fails to prevent or delay the onset of scurvy in guinea-pigs.

Decitrated lemon juice of the same reaction kept for 24 hours at room temperature in the absence of air shows no deterioration in its antiscorbutic potency.

There is no change in the hydrogen-ion concentration of the alkaline decitrated juice kept in the absence of air after 24 hours; when, however, it is exposed to the air there is an increase in the hydrogen-ion concentration.

When air is aspirated through a boiling solution of decitrated lemon juice for one hour the destruction of the antiscorbutic factor is delayed when the hydrogen-ion concentration is raised from p_H 6·6 to 2·2.

The expenses of this research were defrayed from a grant made by the Medical Research Council, to whom my thanks are due.

REFERENCES.

Dutcher, Harshaw and Hall (1921). *J. Biol. Chem.* **47**, 483.
Harden and Zilva (1918). *Lancet*, ii, 320.
Hess and Unger (1919). *J. Biol. Chem.* **38**, 293.
Hess and Unger (1921). *Proc. Soc. Exp. Biol. Med.* **18**, 143.
Holst and Frölich (1912). *Z. Hyg.* **72**, 1.
La Mer (1922). *Dissertation*, New York.
Zilva (1921). *Lancet*, i, 478.

XLVII. A NOTE ON THE CONSERVATION OF THE POTENCY OF CONCENTRATED ANTISCORBUTIC PREPARATIONS.

By SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute, London.

(*Received May 14th, 1923.*)

Some years ago it was shown that decitrated lemon juice prepared in concentrated form was of therapeutic value in the treatment of infantile scurvy [Harden, Zilva and Still, 1919]. The advantage of such a treatment is that very potent antiscorbutic solutions can be administered in very small bulk without introducing at the same time most of the extraneous matter present in active juices derived from natural products. During the last few years the writer had had the opportunity of preparing on a number of occasions concentrated decitrated lemon juice solutions which were used with great success in cases of acute infantile scurvy. These preparations were found to be especially of great value when the patients were suffering from gastric disturbances which made the administration of ordinary antiscorbutics difficult. As an example of this one may quote a recent case of Dr G. F. Still at the Hospital for Sick Children, Great Ormond Street, to whom I am indebted for the following particulars.

Patrick M..... Age 10/12. Admitted Jan. 29, 1923.

Tenderness of limbs since November. Fed on Allenbury foods, No. 1 till 4 months, No. 2 till 7 months, No. 3 (made with milk) until December when he was put back during the last 2 or 3 weeks to No. 2. Has also had some oatmeal water but nothing else.

On admission did not move arms or legs. Screamed with pain when arms or legs touched, especially wrists and ankles; some thickening just above ankles and wrists; apparently periosteal haemorrhage; haemorrhages into gums around incisors; urine, no blood; temperature varying up to 103·4° F.

Treated with potato cream and orange juice; one teaspoonful of each twice a day.

After 6 days, child more comfortable but temperature irregular.

Orange juice given four times daily. By February 5th stools became frequent, green, and loose, and from this time, although the child became much less tender, the looseness of the bowels, with much mucus, continued; there was no naked eye blood in the stools.

By February 19th the child was looking very bad; temperature 104°, eyes sunken, and looked as if likely to die. The ordinary antiscorbutics had been stopped on account of the bowel condition.

Concentrated decitrated lemon juice begun on February 21st; temperature reached normal on February 22nd, rose to 101° F. on 23rd and then fell to normal or thereabouts; the stools steadily improved.

The concentrated lemon juice was continued until March 5th ʒ iii per diem, except from February 21–25 when ½ oz. was given daily; on the first day of an eight times concentrated solution (of which 2 oz. = 15 lemons), and on the second day a ten times concentrated solution, ½ oz. was given.

The child continued to do well and gained weight during the next four weeks during which the periosteal thickenings gradually diminished until they had almost gone altogether.

No further trouble occurred, except that there was a gastro-intestinal disturbance a few days before the child was discharged from the Hospital on April 12th, but this did not appear to be connected in any way with his scurvy.

Such concentrated decitrated solutions, however, deteriorate in potency on keeping and consequently must be freshly prepared before administration—a circumstance which militates against their general use in medical practice. In view of the observations made by the writer on the inactivation of the antiscorbutic factor, conditions have been worked out which make it possible to store such concentrated antiscorbutic solutions without detriment to their activity. As it was found [Zilva, 1923] that destruction of the antiscorbutic factor is favoured by alkalinity and exposure to air, the concentrated antiscorbutic solutions were acidified and stored under anaerobic conditions. A concentrated juice stored in this way did not show any perceptible loss in potency after storage for three months.

Two and a half litres of lemon juice were treated with an excess of calcium carbonate and three volumes of absolute alcohol and filtered. The filtrate was concentrated to a tenth of its volume and acidified slightly. It was then stored in an ordinary conical flask in an evacuated bell-jar over alkaline pyrogallic acid for three months. At the commencement of the experiment a suitable quantity of the concentrated juice was diluted to its original volume and tested out on guinea pigs in order to determine its minimum protective dose. During the period of testing (about six weeks) the diluted juice was also kept under an evacuated bell-jar over alkaline pyrogallic acid, the necessary dose being removed daily. The concentrated and diluted preparations were kept at ordinary room temperature. At the end of three months another quantity of the concentrated preparation was diluted and tested out in the same way as at the commencement. As anticipated no loss of potency was observed. In both cases a daily dose of 1·5 cc. of the preparation which was previously diluted to its original volume afforded protection, whilst daily doses of 0·5 cc. and 1 cc. of both the original and stored preparation only protected the animal partially, the onset of the disease and death being delayed.

Adopting the above principle a method is now being worked out for the preparation of stable concentrated antiscorbutic solutions which could find practical application in the prophylaxis and treatment of infantile and adult scurvy and which it is hoped will be of use to the clinician.

The expenses of this research were defrayed from a grant made by the Medical Research Council to whom my thanks are due.

REFERENCES.

Harden, Zilva and Still (1919). *Lancet*, i, 17.
Zilva (1923). *Biochem. J.* **17**, 410.

XLVIII. ARTIFICIAL PARTHENO-GENESIS IN SEA URCHINS.

A NOTE ON AN IMPROVED TECHNIQUE.

By ISAAC FROST.

From the Department of Physiology, University of Birmingham.

(*Received May 4th, 1923.*)

Working at the Marine Biological Laboratory, Plymouth, at Easter, 1921, the writer followed Shearer and Lloyd [1913] in their modification of Loeb's [1913] technique for the induction of artificial partheno-genesis in sea urchin (*Echinus esculentus*) eggs.

The eggs were shaken into a bowl of fresh "outside" sea water and, if found of the right maturity, were transferred in turn by pipetting into a series of bowls containing the following solutions in the order given, and for the times stated:

(1) 0·3 cc. *N*/10 butyric acid + 50 cc. sea water. 1·5 minutes.
(2) Sea water. Wash } 15 minutes.
(3) ,, ,, }
(4) 0·2 cc. *N*/10 NaOH + 50 cc. sea water. 6 minutes.
(5) Sea water. Wash.
(6) 8·0 cc. 2·5 *M* NaCl + 50 cc. sea water. 45–60 minutes; or 25–30 minutes.
(7) Sea water. Wash.
(8) Final transfer into normal sea water.

These manipulations certainly resulted in segmentation of the eggs, and in the production of swimming blastulae. But the yield varied enormously from experiment to experiment; and, where it was necessary for other purposes to look for mass production, the above method, at any rate in the writer's hands, failed to give consistently good results. Conditions inherent in the egg could not account for the differences. For, specimens of eggs from the same batch treated in the same way at the same time displayed widely differing mortality. Plainly, the discrepancy crept in from outside. Errors in the composition of the various fluids would offer a sufficient explanation.

The pipette method of transfer seemed the most likely source of such error. At each transfer at least 50 %—more often 90 %—of the eggs remain behind in the bowl. On the former generous scale of diminishing returns, where there are at least eight transfers, the last bowl will contain $\frac{1}{2^8}\left(\frac{1}{256}\text{th}\right)$ the number of eggs treated in the first bowl. To obtain any visible number of eggs in the end bowl, it was found that at least 3·0 cc. of sea water containing a thick emulsion of eggs had to be used to commence with.

Now, of what use is it to prepare, meticulously, the exactly proportioned solution containing *e.g.* 0·2 cc. *N*/10 NaOH in 50·0 cc. sea water when an immediate contamination of 3·0 cc., that is 6·0 % is involved in the mere transfer? More often, however, 6·0 cc. or even 9·0 cc. (18 % error) is transferred, or else it is quite impossible to pick out the eggs in the eighth bowl. One has to search for the number of eggs in $\frac{1}{256}$ of 3·0 cc. scattered in a solution of 50·0 cc. sea water. Plainly, at least 6 % error, and more often 12 % to 18 % error, is involved in each transfer. Here then, we may say we have discovered the source of the differing end-results.

The remedy is simple. The experiment must be so arranged that the eggs remain in the same vessel throughout; the changes of medium being effected by drawing off the fluid.

A deep funnel, with a securely fitted filter answers the purpose. The writer adapted the funnel of a Cona coffee still; the filter pad is firmly applied to the bottom of the funnel by means of a long wire passing through the limb of the funnel. A screw at the bottom of the wire serves to tighten the filter pad as much as desired.

The filter pad itself consists of a perforated metal or porcelain disc covered with several layers of fine muslin sewn into the disc. Layers of fine filter paper may be super-added if it is found that the eggs pass through.

A piece of rubber tubing, supplied with a tap, is fitted to the end of the limb, and in this way it is possible to permit the action of a solution for any length of time before withdrawing. Draining off can be effected much more rapidly by the judicious and gentle use of a vacuum water pump, to which the apparatus can be easily attached by rubber tubing. A further advantage in this method is that the eggs can be easily washed any number of times by flushing the apparatus through with sea water.

In conclusion, it may be said that the method is suitable not only for the partheno-genetic treatment of eggs, but for all experiments in which eggs, fertilised or unfertilised, are to be exposed to the action of various fluids.

SUMMARY.

(1) The "pipette" method of transferring eggs from one bowl to another is laborious, time consuming and involves a minimum error of 6 %; more often 12 %–18 %.

(2) The new "funnel" method described is easily adapted to laboratory material, involving the use of a flat-bottomed funnel with perforated plate, muslin and filter paper. A ready made efficient substitute is indicated in the funnel of the "Cona" coffee brewing apparatus.

(3) The method obviates the errors of transfer; it is simple, rapid and involves no eye-strain.

REFERENCES.

Loeb, J. (1913). Artificial Partheno-genesis and Fertilisation, Chicago.
Shearer and Lloyd (1913). *Quart. J. Mic. Sci.* **58**, 523.

XLIX. A SIMPLIFIED METHOD OF PREPARATION OF THE BEZSSONOFF REAGENT FOR VITAMIN C AND SOME POLYPHENOLS.

By NIKOLAI BEZSSONOFF.

From the Laboratoire de Chimie Biologique de Colombes, France.

(*Received May 18th, 1923.*)

In 1921, I described [1921] a blue coloration common to antiscorbutic extracts, to quinol and to catechol and obtained in acid medium with a phosphomolybdotungstic acid having the formula: 17 WO_3 (MoO_3) (P_2O_5) 25 H_2O.

Concerning the reaction of this acid in the presence of antiscorbutic extracts, I noted the absence of a blue coloration with plum juice which is a juice devoid of any antiscorbutic action and on the contrary the presence of this coloration with the peach, though belonging to the same genus as the plum. I therefore concluded that the peach contained the antiscorbutic, although this fruit had not at that time been examined by animal tests. Since then Delf [1922] has verified the antiscorbutic action of the peach.

It seems then that this reagent is of value in the study of the antiscorbutic value of plants which have not been examined in the usual way.

It could equally serve to reveal the presence of polyphenols in different biological liquids (urines, etc.).

It seems useful to indicate a quick and simple method in order to obtain this reagent in a crystalline state and sufficiently pure: 36 g. of sodium tungstate and 4 g. of phosphomolybdic acid are dissolved in 200 cc. of distilled water at a temperature of about 50°; then 5 cc. of 85 % phosphoric acid are added and 10 cc. of concentrated sulphuric acid are poured drop by drop into the above solution which should be stirred as the same time. The solution is evaporated slowly at a temperature of 40–42°. Slowly pale yellow monoclinic crystals are formed at the bottom and on the sides of the vessel. At the end of 20 to 24 hours, the solution being concentrated to about 1/3, the liquid is poured off. In order to obtain a sufficiently pure reagent without proceeding to a new crystallisation, 2 to 3 cc. of distilled water are poured on the crystals. They are then stirred slowly and the liquid is immediately poured off. This operation is repeated until one drop of the water used for washing the crystals gives a blue coloration with a quinol solution or a brown-yellow with a pyrogallol solution (1 in 1000 in each case). The washed crystals are dried between filter paper, then 15 g. are dissolved in 100 cc. of 5 % (by volume) sulphuric acid.

Prepared in this way, the reagent can be preserved two months and more in coloured glass bottles using a ground glass or rubber stopper (avoid a cork stopper).

I recall [Bezssonoff, 1921, 1922] that this reagent must be employed in an acid medium. The sulphuric acid present in the solution generally suffices to secure this condition.

REFERENCES.

Bezssonoff (1921). *Compt. Rend. Acad. Sci.* **173**, 917.
—— (1922). *Bull. Soc. Chim. Biol.* **4**, No. 2, 83.
Delf (1922). *Lancet*, i, 576.

L. STUDIES ON INOSITOL.

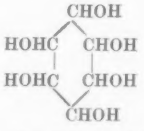
I. A METHOD OF QUANTITATIVE ESTIMATION.

By JOSEPH NEEDHAM (*Benn Levy Student*).

From the Biochemical Laboratory, Cambridge University.

(*Received May 13th, 1923.*)

Inositol was first discovered in 1850 by Scherer [1850], who isolated it from Liebig's extract of meat and accurately determined its empirical formula. Between that date and 1864 it was studied by Cloetta [1856], Müller, W. [1857], Cooper-Lane [1861] and Marmé [1865] who gradually outlined the main features of the problem of its significance, ascertaining its distribution in plants and animals, and elaborating tests and methods of extraction. All the earlier workers regarded it as in constitution allied to the sugars, because of its empirical formula, $C_6H_{12}O_6$; and it was accordingly named "muscle-sugar," from one of its principal sources. Thus it was left for Maquenne to show in 1887 that inositol was not a sugar but a cyclic hexamethylene compound, or hexahydroxyhydrobenzene.



Ever since then, the obvious physiological implications of such a structure have attracted considerable interest, and the more so since the discovery of phytin, the calcium magnesium salt of inositolhexaphosphoric acid, in plants, by Palladin [1895], Winterstein [1897], Posternak [1903], and others. Throughout both periods all investigation has been greatly hampered by the lack of a reasonably accurate method of quantitative estimation, without which no real advance could be made. From this difficulty the plant chemists found a partial way out by determining the relative amounts of inorganic and organic phosphorus, though this could only give the most indirect knowledge of the metabolic significance of inositol. However, as far as the animal body was concerned, in spite of the lack of an estimation-method, Starkenstein [1909] concluded that inositol "has no special physiological significance in metabolism." Rosenberger [1910], from an experimental basis even more doubtful, put forward the contrary view that inositol subserved some most important function.

In view of such facts as these, it seemed desirable to continue the search for a method, as the really essential preliminary to an extended study of the general physiological meaning of the inositols.

ATTEMPTS TO EVOLVE A METHOD ON A NEW PRINCIPLE.

The first observation which seemed to be hopeful was found in the work of Neuberg [1908], who succeeded in obtaining furfural from inositol. Some such procedure as that used in the estimation of pentoses might have been possible, but on looking up the original paper, it was found that the change was anything but quantitative, necessitating dry distillation with phosphoric anhydride, and only yielding very small amounts of furfural. Rosenberger in 1910 had hoped that the hexacetate of inositol prepared by Maquenne in 1887 might be applicable to a quantitative method, but this also seemed useless, because of the tri- and tetra-acetates formed at the same time—at any rate for the minute quantities of inositol found in tissue extracts.

A colorimetric method would have been most desirable so the colour-tests for inositol were examined carefully. The most obvious thing about them is that they all depend on the reactions of the oxidation-products of inositol, rhodizonic acid, and other quinonoid bodies. Scherer's original test [1852], modified successively by Mayer [1907] and Salkoffski [1910], consists essentially of a rapid oxidation with strong boiling nitric acid, followed by the formation of calcium rhodizonate (?) which is brick-red or pink in colour. The reactions of Meillière [1906, 1, 2, 3] and of Gallois [1865], are exactly the same in principle, but the red mercury salt is formed instead; while Perrin's test [1909] depends on the formation of a reddish silver salt. Denigés [1907], after oxidation with nitric acid, adds potassium hydroxide, sodium nitrocyanide, and acetic acid; the solution is said to change from blue to brown to red; Seidel [1884, 1887] reported good results from the use of strontium acetate. Hugo Müller's procedure is the only one which does not utilise oxidation with nitric acid, for here hydrogen peroxide is used instead in the presence of a ferrous salt in traces [1907, 1912].

The writer's experiences with these tests were far from satisfactory. Müller's reaction could be ruled out at once for the purpose in hand, for the products were known to be many and various, oxalic and other acids being produced as well as quinones. In spite of the utmost care it was impossible to get Denigés' test to work at all, and the same applied to the test introduced by Seidel. Seidel's paper was unfortunately inaccessible to the writer, but he was informed by Dr Rosenheim that in the original description of the test, strontium acetate does not appear, and the text-books have described it wrongly. It is surprising that the "Analyse des Harns" of Neubauer and Vogel, usually so accurate, should have fallen into this mistake. What Seidel really used was sodium acetate and barium chloride, in which case the reaction is simply a variant of the Scherer test and is easy to perform.

The Scherer reaction itself, the oldest and most used of all, seemed at first likely to be of some use quantitatively. It appears that a chloride (Ba, Ca, Sr, Al) is necessary, as well as traces of a catalyst such as platinum or a mild oxidising agent like chloramine-T. With the aid of these sugges-

tions and of Mayer's modification, the comparative delicacy of the test was investigated, and it was found that only down to 1 in 3000 were the results uniform and constant. Below that dilution, unknown factors seemed to possess great influence. Furthermore, Mayer [1907] reported failure of the test with a sample of pure inositol, and it was found in this work that quite small quantities of lead salts completely inhibited the reaction. The reaction could not be applied, then, to the estimation of inositol in tissue extracts, or in any even moderately complex mixture.

In consequence, it was necessary to fall back on the classical method for the isolation of inositol; precipitation with basic lead acetate and subsequent decomposition with hydrogen sulphide. This has been used in the past by many workers, including Marmé [1865], who introduced it; Gallois [1864], Külz [1876], Rosenberger [1908, 1, 2] and Starkenstein [1909]. As the basis for a method of estimation it suffered from the fact that it had never been shown to be quantitative, though Meillière [1906, 2] assumed that it was, and Starkenstein [1909] referred to it as "fast quantitativ." Meillière [1907] had reported that the copper acetate compound of inositol studied by Thudichum was insoluble, and could be quantitatively precipitated: the writer's experiences with this are recorded below. In such attempts at quantitative work as had been done, the lead precipitation had been used in the course of "estimation by weighing the isolated product." This of course is an inaccurate procedure, and requires the taking of large amounts of initial material, thus effectively masking any small variations in the tissue; to say nothing of the time consumed in working up the resulting extracts. It therefore occurred to the writer that if the lead precipitation were united to a good method of extraction, and to a micro-carbon estimation at the final stage, some progress might be made with the general problem. The following experimental work was accordingly carried out.

The process of extraction.

All the earlier workers were accustomed to use extractions of hot and cold water. W. Müller [1857] departed from this practice, however, by grinding wet brain tissue with neutral lead acetate, and Rosenberger [1908, 2] by boiling the tissue with potash, and subsequent alternate treatment with nitric acid and baryta. Both methods were most unsatisfactory; the former because it gave very impure samples of inositol, and the latter because of its drastic nature—"any inositol, which has survived this prolonged treatment," as Momose [1916] says, "being precipitated by lead in the usual way." Momose, working on a suggestion of Rosenheim's, discovered that very good yields of inositol from brain tissue were to be obtained by working up the first watery acetone extract. By this means, inorganic salts, known to interfere with the lead precipitation, were much reduced in quantity, and, as very little else besides inositol was extracted, the crude product was purer.

To find whether this procedure was, or could be made, quantitative, 6 kilos. of butcher's beef muscle was carefully freed from fat, finely minced, and extracted with acetone in the proportion of 1·25 litres per kilo. This, of course, amounts to extraction with dilute acetone, since the muscle contains about 70 % water. After standing all night the strained and filtered liquid was evaporated under diminished pressure to remove all the acetone, and the aqueous residue was worked up for inositol in the usual manner, *i.e.* precipitation successively with neutral, then with basic, lead acetate; decomposition with hydrogen sulphide, and finally precipitation with alcohol and ether. During the stages of the process, qualitative tests were made to show the presence and behaviour of other possible extractives; these are given in Table I.

Table I.

	After removal of acetone	After precipitation with neutral lead acetate	After precipitation with basic lead acetate	After addition of alcohol (absolute)
Reducing sugar	–	–	–	–
Inositol	+	+	+	+
Creatinine	+ +	+ +	+	–
Creatine	+	+	–	–
Glutathione	+ +	–	–	–
Glycogen	–	–	–	–
Lactic acid	–	–	–	–
Cholesterol	–	–	–	–
Purine bases	–	–	–	–

Meanwhile the tissue was again extracted by shaking with acetone in the same proportions for about four hours and allowing it to stand thus overnight. The second acetone extract was treated in exactly the same manner as the first, except that 2500 cc. of water was added to compensate for the absence of the tissue water in the first instance. Then the tissue was extracted a third time, but with water. The results appear in Table II.

Table II.

		Amount of inositol isolated in g.
Extract 1.	Acetone and water	0·935
„ 2.	„ „	nil
„ 3.	Water only	nil

It was afterwards found that tissue should not be allowed to remain in the acetone longer than three days; for cholesterol begins then to be extracted in spite of the water present.

It seems justifiable, then, to conclude from these data, that the process of extraction with dilute acetone succeeds in removing from the tissue all the inositol which it contains. The sample obtained in the experiment given above melted at 221° and when examined in the polarimeter, in comparison with a sample of *i*-inositol prepared from phytin, failed to exhibit any optical activity. The conclusion is that the inositols of ox muscle, ox brain, human brain, and phytin, are identical, the last three having been shown to be so by Momose [1916].

THE PROCESS OF PRECIPITATION.

To prepare a supply of inositol, which could be drawn upon for blank experiments, a quantity of commercial phytin was procured and hydrolysed under pressure, bearing in mind the experiences of Plimmer and Page [1913] with organic phosphorus esters. Into hard glass tubes were placed 50 g. of phytin, with 200 cc. of 35 % sulphuric acid; these were sealed and heated for nine hours at 170°. Afterwards, the magnesium and calcium sulphates were filtered from the reaction mixture, the sulphuric acid removed as barium sulphate, excess of baryta as barium carbonate, and the inositol brought down by the addition of alcohol. From 50 g. of phytin, 8·4 g. of inositol were obtained; or about 84 % of the theoretical yield, assuming that Anderson's formula for phytin is correct [1912]. This inositol (M.P. 219°) was used for the following series of experiments. Since, as has been noted above, traces of lead interfere with the Scherer reaction, it was not possible to depend on it as an indication of the presence of inositol, and in each case it was necessary to evaporate the solutions to low bulk and precipitate the inositol, if it was present, with absolute alcohol or ether.

In order to approximate as much as might be to the actual conditions of tissue extracts, the concentration was made about the same as it would be expected to be from 200 g. of muscle. 8 cc. of a standard 0·5 % inositol solution were diluted to 140 cc. with distilled water, thus allowing for the water of the tissues. To this the saturated basic lead acetate solution was added, the conditions being varied. A preliminary test was done to see if the addition of 500 cc. of acetone, and its removal, had any effect on the inositol content. *A priori*, this was most unlikely, as inositol is insoluble in acetone; and colour-tests showed that it was not the case.

Table III.

	Inositol found in the filtrate after decomposition of lead compound with H_2S and precipitation by alcohol
Basic lead acetate, saturated solution boiled	
equal quantity, or less	+ + +
excess	+ +
excess + concentrated ammonia 3 cc.	+ +
excess, reprecipitated 3 times	+ +
excess left to stand three days	+ + +
Basic lead acetate, saturated solution unboiled	
equal quantity, or less	+ –
excess	+ –
excess + concentrated ammonia 3 cc.	–
excess + reprecipitated three times	–
excess left to stand three days	+ +

Inositol tends to be held, perhaps in an adsorbed state, by the precipitate of lead sulphide, requiring two extractions with water at about 70° to remove it completely. It is readily adsorbed by Fuller's earth, though not by kieselguhr. Several points are clear from the above results. In the first place, Momose's observation that the lead acetate compound dissociates on prolonged washing receives corroboration from the fact that the precipitation

seems to fail when the mixture is not filtered for three days. The ordinary hydrolysis of the basic salt to the hydroxide is presumably much intensified by boiling, hence the importance of using a cold unboiled saturated solution. Meillière showed that the presence of ammonia was important in the precipitation, and his observation was fully confirmed. The quantities of inositol appearing on the addition of absolute alcohol were too small to be weighed; and when no trace of cloudiness appeared, the result was recorded as negative.

To effect a further separation the well-known insolubility of inositol in alcohol was made use of. By evaporating to low bulk (less than 20 to 30 cc. if quantities of the order of 40 mg. as here, are present) and the addition of 300 cc. of absolute alcohol, the inositol present is all precipitated, and the resulting slight cloudiness flocculates well after half-an-hour's standing. In this connection the statement of Cooper-Lane [1861] could not be confirmed. He considered that if too much alcohol was added, the inositol failed to come out of solution; but this was never found to be the case if the watery solution was taken down to a sufficiently low bulk. Reprecipitation after adding a little water to the filtered liquid, and distilling off the alcohol, never gave any further cloudiness.

After standing overnight to ensure complete precipitation of the inositol, it is filtered off from the alcohol by passing it through a carefully prepared Gooch crucible, with very small holes, containing a layer of the cleanest asbestos about three millimetres thick. The air current drawn through by the filter-pump is allowed to pass for 10 minutes to make sure that the asbestos and inositol are quite free from alcohol; and the content of the crucible is then quantitatively transferred to a 500 cc. flask in which it is shaken with about 60 cc. of distilled water. At the end of 10 minutes the inositol is completely dissolved and the asbestos is then filtered off. The solution contains all the inositol from the tissue taken—and nothing else—and, after being made up accurately to 100 cc. is ready for estimation by the micro-carbon part of the method. Numerous tests, carried out on the various stages of this part of the whole method, showed that the inositol could be transferred thus from alcohol to water, without the least loss.

The lead precipitation described above proved satisfactory and could always be relied on. Thudichum [1864], considered precipitation by copper acetate to be quantitative if the inositol solution was evaporated to low bulk on a water-bath with about twice its volume of saturated copper acetate solution, until no more of the green flocculent precipitate was formed. The degree of accuracy which this method possesses can be gauged from the following typical example:

Calculated amount of inositol in the solution per cc. = 25 mg.
Found by the copper precipitation = 24·2 mg.

The method gives low results when checked against the lead precipitation, and is more limited in its application than the latter, since it fails in the presence of traces of sugar.

The process of microestimation of carbon.

Faced with the problem of estimating these small quantities of inositol in pure solution, it occurred to the writer that the quickest way of proceeding would be to ascertain the carbon-content by means of a micro-combustion method, and to calculate the inositol content from that. Evaporation and subsequent weighing in a platinum dish would of course have solved the difficulty, but the time required in drying to constant weight seemed to contraindicate this method. The method used [see Needham, 1923], cannot be termed a wet combustion in the strict sense, for that would imply one of the chromic acid oxidation methods; what was done was to evaporate a known amount of the solution in the actual boat of the combustion furnace.

The micro-methods collected and described by Pregl [1917] and others, were here of little use, for they all apply to combustions of the dry material, and to deal quantitatively with such small amounts of it as 20 mg. or less, would be very difficult. An expensive microchemical balance would be necessary for the preliminary weighing out, and the final measurement, being gravimetric, would estimate such small quantities of carbon dioxide less well than a burette measurement. These difficulties were overcome by the aid of a method of combustion from solution, and the measurement of the gas evolved, by volume.

In order to check the accuracy of the whole process, from beginning to end, it was necessary to carry out all the operations on a solution of known strength. For this purpose 8 cc. of a 0·4 % solution of inositol were taken, diluted to 140 cc. and the whole method proceeded with as described above. The calculated concentration of inositol in the final pure aqueous solution amounted to 32·00 mg. %. From 1 cc. of the liquid 0·231 cc. of CO_2 were given off on combustion. This, when translated into terms of concentration of inositol, is equivalent to 31·70 mg. %.

The method applied to some tissues of the rabbit.

Two rabbits, *A* and *B*, *B* freshly killed, and *A* dead two days before, were taken and estimations performed as described: on thigh-muscles, heart, kidney and liver. The following figures were obtained:

				mg. %		
Tissue	Rabbit	Cc. of CO_2 finally evolved	Tissue originally taken in g.	Of final solution	Of original tissue	g. per kilo
Muscle	*A*	0·241	100·0	16·1	16·1	0·16
„	*B*	0·171	121·0	11·5	9·3	0·10
Heart	*A* and *B*	0·234	15·5	10·9	100·6	1·00
Liver	*B*	0·209	85·5	15·6	16·3	0·16
Kidney	*A* and *B*	0·163	35·5	14·0	30·6	0·30

Until more extended investigations have been made, it is impossible to appraise the exact significance of the above figures. For example, the individual variations of normal rabbits are as yet unknown. At the same time, while bearing this in mind, it is certainly interesting that the muscle from the

freshly-killed rabbit contained 50 % less inositol than that from the rabbit which had been dead two days. It recalls the view of Rosenberger [1908, 1, 1910], that tissues contain an "inositogen"; though his conclusions were drawn from the comparison of fresh rabbit muscle, and commercial beef; tissues perhaps hardly comparable. Unfortunately, there are no previous figures with which to compare the above. But it is worth remembering that Marmé [1864], reported specially good yields of inositol from heart muscle.

Obviously, the applicability of the method as a whole, may not be general. In extracts of plant tissues, for example, or of animal tissues, not yet investigated, there might well be substances present, which, extracted by dilute acetone, and carried down by basic lead acetate, would seriously affect the final result. But as far as the data go at the time of writing, the general principle seems safe.

Summary.

1. A method is described for the estimation of *i*-inositol in animal tissues.

2. Preliminary results, obtained by the aid of the method on some of the tissues of the rabbit, are given.

3. The inositol of ox muscle is identical with that which is produced by the hydrolysis of phytin.

The writer wishes to take this opportunity of thanking Professor Hopkins for his interest in the work. He acknowledges also with gratitude much advice received from Mr J. B. S. Haldane on gas analysis, and from Dr O. Rosenheim on the chemical behaviour of inositol. Mr Morven Busby rendered valuable assistance with certain of the experiments. Lastly, the writer's thanks are due to the Clayton Aniline Company, representing the Basel Society of Chemical Industry, who were good enough to supply the phytin used in the work.

References.

Anderson (1912). *J. Biol. Chem.* **11**, 475.
Cloetta (1856). *Liebig's Annalen* **99**, 302.
Cooper-Lane (1861). *Liebig's Annalen* **117**, 118.
Denigés (1907). *Comptes Rend. Soc. Biol.* **62**, 101 and 507.
Gallois (1864). L'Inositurie, Paris.
—— (1865). *Z. angew. Chem.* 264.
Külz (1876). *Maly*, 45.
Marmé (1865). *Liebig's Annalen* **129**, 222.
Mayer (1907). *Biochem. Z.* **2**, 393.
Meillière (1906, 1). *Chem. Zentr.* 1528.
—— (1906, 2). *Comptes Rend. Soc. Biol.* (1), 226.
—— (1906, 3). *J. Pharm. Chim.* (6) **24**, 241.
—— (1907). *J. Pharm. Chim.* **26**, 300.
Momose (1916). *Biochem. J.* **10**, 120.
Müller, H. (1907). *J. Chem. Soc.* **91**, 1780.
—— (1912). *J. Chem. Soc.* **101**, 2383.
Müller, W. (1857). *Liebig's Annalen* **103**, 140.

Needham (1923). *Biochem. J.* **17**, 431.
Neuberg (1908). *Biochem. Z.* **9**, 551.
Palladin (1895). *Z. Biol.* **31**, 199.
Perrin (1909). *Ann. Chim.* **14**, 182; *Chem. Zentr.* 564.
Plimmer and Page (1913). *Biochem. J.* **7**, 157.
Posternak (1903). *Comptes Rend. Acad. Sci.* 137, 202, 337, 439
Pregl (1917). Mikro-analyse, Berlin.
Rosenberger (1908, 1). *Z. physiol. Chem.* **56**, 373.
—— (1908, 2) *Z. physiol. Chem.* **57**, 464.
—— (1910). *Z. physiol. Chem.* **64**, 341.
Salkoffski (1910). *Z. physiol. Chem.* **69**, 478.
Scherer (1850). *Liebig's Annalen* **73**, 322.
—— (1852). *Liebigs' Annalen* **81**, 375.
Seidel (1884). *Dissert. Univ. Dorpat.*
—— (1887). *Chem. Zeitung*, **11**, 676
Starkenstein (1909). *Z. exp. Path. Ther.* **5**, 378.
Thudichum, W. (1864). The Chemical Constitution of the Brain, London.
Winterstein (1897). *Ber. deutsch chem. Ges.* **30**, 2299.

LI. A NOTE ON THE ESTIMATION OF THE CARBON-CONTENT OF SOLUTIONS.

By JOSEPH NEEDHAM (*Benn Levy Student*).

From the Biochemical Laboratory, Cambridge University.

(*Received May 13th, 1923.*)

The method to be described in this note was evolved to meet a special case, namely, the estimation of very small quantities of a carbon-containing compound in pure solution. In the first of a series of papers on inositol [Needham, 1923], the causes which led to the working out of the method are discussed, and it will be sufficient to say here that it was desired to know the carbon-content of amounts of solution containing as little as 20 mg. or less of *i*-inositol. But the method has also proved applicable to any solution of unknown carbon-content, and has been used successfully by other workers in the laboratory in dealing with bacterial culture media, etc. [Stephenson and Whetham 1923].

The method is not a wet combustion in the usual sense, for that would imply oxidation with chromic or sulphuric acid and weighing the gas evolved, as in the method of Tangl and Kereszty [1911]. Rather it is a combustion from solution, for an accurately measured out quantity of the liquid is evaporated from the receptacle in which the combustion is to take place. A wet combustion in the strict sense would not have been so useful in the case of inositol, because of its great stability towards mineral acids, but in some cases would be no doubt preferable. The sulphuric or chromic acid methods appear to possess no advantage in the matter of time and although the use of a silica tube is avoided, yet a special and somewhat complicated piece of apparatus is necessary for the carrying out of the decomposition.

The central point of the apparatus is a quartz combustion tube filled in the usual manner with rolls of copper gauze and a quantity of copper oxide in wire-form. The boat is of silica and may vary in size; into it is run from an accurate micro-burette a known amount of the solution to be estimated. Throughout the process of combustion, which is over in 20 minutes, a stream of oxygen is passed through the apparatus. A wash-bottle of concentrated sulphuric acid, towers containing soda-lime and sticks of dry potassium hydroxide, and a baryta wash-bottle to indicate the presence of any trace of carbon dioxide come between the oxygen-source and the furnace. On the other side of the furnace the gases first pass through a bulb to collect the condensed water, furnished at the bottom with a tap so that after every estimation the water produced can be run off, and no risk incurred of its

absorbing carbon dioxide in subsequent experiments. True, it could absorb a small amount during the combustion which produced it, but this danger is the less, as, having just been condensed from the state of vapour, it is hot. Next, the gases pass through a bulb containing sulphuric acid, and a U-tube filled with calcium chloride. Between these and a long cooling coil there is placed a screen about five feet square, made of wood, and covered with thick asbestos on both sides. After the cooling coil comes a 2-way tap which can direct the gas either through an index-bubbler or through the absorption apparatus. This, shown in greater detail in the appended figure, is an ordinary

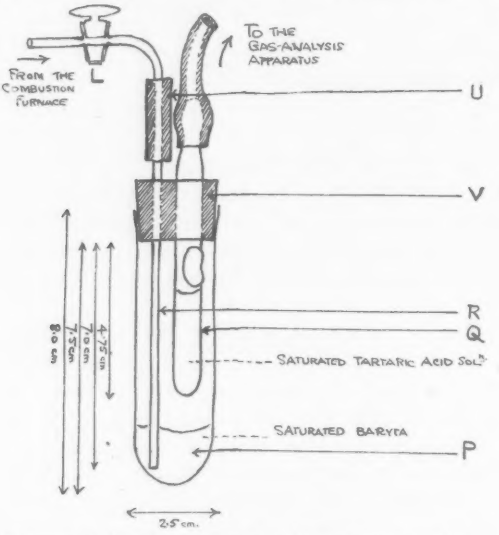


Diagram of the absorption-bulb in the combustion method.

Total internal capacity of bulb =		32·5 cc.
Space occupied by tubes	= 4·0 cc.	
,, ,, ,, baryta	= 5·0 cc.	
,, ,, ,, acid	= 1·0 cc.	
	10·0 cc.	10·0 cc.
Value employed in solubility correction		22·5 cc.

large thin glass test-tube, drawn out and rounded, and is attached to a Haldane gas measuring apparatus, made exactly as described by Haldane [1920]. The tube connecting with the furnace has another tap through which the gases are led on down the tube *R* to the bottom of the absorption bulb, through the rubber cork *V*. *U* is a collar of rubber tubing to permit of a firm hold for the crucible-tongs when the bulb is shaken in the process of evolving the gas. Exactly 5 cc. of saturated baryta are placed in the bottom of the flask as shown in the diagram so that the tube *R* dips down into it. The tube *Q*, containing 1 cc. of a saturated solution of tartaric acid, which connects with the measuring burette, is pierced by an aperture just underneath the

cork *V*, large enough to permit an easy shaking out of the tartaric acid, and sufficiently small to prevent any escaping before the proper time. The carbon dioxide, then, produced by the combustion of the carbon-containing compound, is dried, cooled, and caught by baryta in such a manner that it can subsequently be driven off and measured. Since it is one of the heavier gases, it tends to collect at the bottom of the cooling coil, but it is readily washed out from there by a rapid stream of oxygen passed through at the conclusion of the combustion.

First, the blank of the whole apparatus was determined. In all cases whether blank or not, the procedure was exactly the same. The results here given are corrected for solubility of CO_2 in the saturated barium tartrate solution, and reduced to N.T.P. Haldane describes the corrections in full.

Blank experiments (empty boat, or boat with distilled water).

Exp. No. IV	12	0·082 cc. of gas evolved	
	13	0·070	,, ,,
	14	0·075	,, ,,
	16	0·090	,, ,,
	18	0·102	,, ,,
	19	0·091	,, ,,
	20	0·084	,, ,,
	21	0·095	,, ,,
	22	0·088	,, ,,
	Average =	0·081	

This blank value was redetermined from time to time, and grew more constant as the apparatus was used. Its high figure cannot be accounted for by traces of CO_2 or by the "reagent blank" (no oxygen stream or heating) for the latter is very constant at 0·015. If oxygen is passed through without heating the combustion tube, but keeping all the other conditions constant, the blank is still at 0·015, so the most probable supposition is that small quantities of carbon monoxide from the oxygen cylinder are burnt in the tube and appear as carbon dioxide. An iodine pentoxide tube would have avoided this, but the constancy of the blank was thought to render it unnecessary.

The next thing that was done was to place in the tube accurately measured out amounts of a solution of known strength of some pure carbon compound and carry out combustions on them. Inositol was chosen.

0·1 cc. of a 0·5 % solution of inositol (equivalent to 0·5 mg. inositol); gas evolved in cc. 0·333, 0·391, 0·408, 0·370, 0·382; average = 0·377; calculated value = 0·374.

0·2 cc. of a 0·5 % solution of inositol (equivalent to 1 mg. inositol); gas evolved in cc. 0·761, 0·718, 0·716; average = 0·730; calculated value = 0·758.

The micro-combustion method was then checked against the evaporation of the final solution, first in a porcelain basin, and then in a platinum dish; followed by weighing the residue before and after incineration. The figures of a typical example are as follows:

From 5 cc. of solution, there were evolved $\begin{cases} 0{\cdot}310 \text{ cc. of gas.} \\ 0{\cdot}326 \quad ,, \end{cases}$

The solution therefore contained 9·19 mg. of inositol %.

Weight of platinum dish		24·5300 g.
,, ,,	after evaporation to dryness	24·6020 g.
,, ,,	after incineration	24·5928 g.
Weight of organic content		9·2 mg.

This method has been used to obtain preliminary data concerning the inositol content of the tissues of rabbits: the figures are given in the other paper.

As against the process of evaporating to dryness and drying to constant weight, it possesses great advantage in the matter of time. From first to last the method of combustion from solution requires only a little over half an hour, whereas the alternative procedure demands a very much longer wait.

It is to be hoped that the method above described will prove useful in the future to workers desiring to estimate the carbon-content of quantities of liquid containing less than 20 mg. of the carbon-containing substance per 100 cc.

REFERENCES.

Haldane (1920). *J. Path. Bact.* **23**, 443.
Needham (1923). *Biochem. J.* **17**, 422.
Stephenson and Whetham (1923). *Proc. Roy. Soc.* B
Tangl and Kereszty (1911). *Biochem. Z.* **32**, 266.

LII. THE EFFECT OF INSULIN ON THE GLYCOGEN IN THE TISSUES OF NORMAL ANIMALS.

By HAROLD WARD DUDLEY and GUY FREDERIC MARRIAN.

From the Department of Biochemistry and Pharmacology, National Institute for Medical Research, Mount Vernon, Hampstead, N.W.

(*Received May 24th, 1923.*)

The immediate and most striking effect of insulin when injected into normal animals, is the fall of blood sugar.

Experiments made by one of us in collaboration with Laidlaw, Boock and Trevan [1923] suggest that this disappearance of sugar is not due simply to an increased combustion of carbohydrate, since the output of carbon dioxide and the oxygen consumption of normal animals under the influence of insulin both fall continuously and rapidly to a very low level, while the respiratory quotient, after a short, transient rise also falls. Kellaway and Hughes [1923] studying the normal human subject, which behaves in a similar way, consider that the glucose disappears, not because it is burnt, but rather because it is converted into some complex substance.

The recent suggestion of Dale [1923] that the action of insulin is to shift the incidence of combustion on to the carbohydrate and to inhibit or at any rate spare the normal catabolism of fats and proteins must be borne in mind and experiments to test this hypothesis are being performed.

The evidence, as far as it goes, being rather in favour of the view that the vanished glucose has not left the body as carbon dioxide and water, it was anticipated that possibly the effect of insulin might be to favour the laying down of glycogen by the liver, as is apparently the case in diabetic (depancreatised) dogs [Banting and colleagues, 1922], and that consequently the glucose of the blood might be found as glycogen in that organ. But on putting the matter to the test, it was found that not only is the sugar not stored in this manner, but the livers of normal animals which have received sufficient insulin to bring them to the verge of hypoglycaemic convulsions contain very little glycogen. It appears, in fact, that as soon as the blood sugar falls to a certain level the liver mobilises its glycogen and pours glucose into the blood stream, in an effort to maintain an adequate concentration of sugar there.

The possibility of a transference of glycogen from the liver to the skeletal muscles and a temporary storage of glycogen in them was considered and

investigated. Again, as in the case of the liver, a disappearance and not an accumulation of glycogen was observed. The experiments on this question seem to indicate that the glycogen of the muscles remains more or less unaffected until that of the liver is greatly depleted, and that then the muscle glycogen is called upon to provide glucose for the circulating blood.

The heart muscle, as might be expected, appears to retain its glycogen to the last. It may be mentioned that the fat content of the livers of mice which had been given large doses of insulin was compared with that of the livers of normal mice. No difference either in the quantity of fat or its degree of unsaturation, as indicated by iodine values, was detected. Apparently, then, the effect of insulin is not to cause a conversion of carbohydrate into fat. Further experimental work on the fate of the sugar in normal animals under the influence of insulin is being carried out.

Experimental.

The glycogen content of the livers of normal mice and of mice after insulin.

Three normal mice were killed, the livers rapidly removed and worked up separately by Pflüger's method. The sugar in the hydrolysed fluid was estimated by Shaffer and Hartmann's micro-method [1920].

Weight of mice in g.	Weight of livers in g.	% glycogen in liver
24	1·79	2·02
21	1·47	2·36
18	1·22 (cyst in liver)	0·89

Three mice were each given 1/400 mg. of "insulin picrate" [Dudley 1923] subcutaneously, and were killed immediately on the appearance of convulsions, which happened within an hour of the injection.

Weight of mice in g.	Weight of livers in g.	% glycogen in liver
23	1·17	
21	1·15	0·075
20	1·12	

As preliminary qualitative experiments had shown the disappearance of glycogen from the livers of such mice the three livers were combined and worked up together.

It will be seen that the average glycogen content of these livers is less than one-tenth of the lowest of the three control animals. It should be noted that there was a large cyst in the liver of the third control animal; this may account for the glycogen content of its liver being so much below that of the other two controls, whose livers were quite normal.

This experiment has been performed several times and the result is always the same. A quantitative determination of the glycogen is not necessary to convince one of the main fact of the disappearance of glycogen under the

influence of insulin. On precipitation of the glycogen with alcohol it is at once strikingly apparent that the glycogen of the insulin-treated livers is much less than that of the normals.

Glycogen in the liver and muscles of the normal and insulin-treated rabbit.

Two rabbits, which had been well fed on carrots and beetroot for a couple of days previous to the experiment were used in this experiment. One of the rabbits, weighing 2·3 kilo. received an injection of 10 mg. (10 2-kilo. rabbit units) "insulin hydrochloride" [Dudley, 1923]. It developed hypoglycaemic convulsions six hours after the injection. It was then killed. The other rabbit, weighing 2·8 kilo., which had been kept in a cage without food from the time of the injection of the first rabbit, was killed at the same time. The livers, hearts, and about 50 g. of thigh muscle, were removed from the animals promptly and worked up for glycogen according to Pflüger's method.

The following results were obtained:

	Normal rabbit % glycogen	Insulin rabbit % glycogen
Liver	5·53	1·86
Heart	0·26	0·54
Skeletal muscle	0·57	0·0

It will be noted that the glycogen content of the insulin rabbit's liver is much less than that of the normal, and there was so little glycogen in the insulin rabbit's muscle that it could not be estimated.

The glycogen content of the normal rabbit's heart was only about half that of the insulin rabbit's, but no special significance is attached to this result, since it is known that such differences do occur in normal animals [Abderhalden, 1914], and another determination on a normal rabbit's heart gave 0·57 % glycogen.

The fat content of the livers of normal mice and of mice after insulin.

Thirteen normal mice with an average weight of 20·7 g., the extremes being 25 g. and 18 g., were killed by first stunning and then bleeding by cutting the vessels of the throat. The livers, bulked together, weighed 14·91 g. The tissue was hydrolysed in 60 % KOH on a boiling water-bath, a stream of hydrogen being passed through the flask to prevent oxidation of the unsaturated fatty acids. The hydrolysed liquid was strongly acidified with HCl and the fatty acids were shaken out with ether. The ethereal solution was thoroughly washed with water and then evaporated to dryness *in vacuo*, a brownish crystalline mass being obtained. This was dissolved in 50 cc. of absolute alcohol.

Thirteen mice with an average weight of 21·5 g., the extremes being 24 g. and 19 g., were injected subcutaneously with insulin hydrochloride (1/1000 mg. per gram of mouse) and placed in a room warmed to 28°. In three-quarters of an hour most of the mice were dead and the rest moribund. Their livers were

collected and weighed 18·95 g. The fatty acid was extracted by the same technique as in the case of the normal mice livers and the resultant fatty acid was dissolved in 50 cc. of absolute alcohol.

10 cc. of each of the solutions of fatty acid were titrated with alcoholic *N*/10 KOH using phenolphthalein as indicator and the amount of KOH required was practically identical in each case; namely, 5·4 cc. for the control and 5·32 cc. for the insulin experiment.

The remaining 40 cc. of alcoholic solution were in each case evaporated to dryness and the residues were taken up in chloroform.

Iodine values were determined by Hübl's method. There was no significant difference in the volumes of $Na_2S_2O_3$ required to titrate the excess iodine.

A comparison of the weights of the liver taken in these experiments is not justifiable since the insulin livers, which weighed considerably more than the normal livers, were obviously gorged with blood. The total weight of the normal mice was 269·1 g. and that of the insulin mice was 279·5 g. It is therefore a fair assumption that the total weight of liver tissue taken in the two experiments was the same within, say, 5 %. Therefore, as the amounts of fatty acid obtained were practically identical in both cases, the experiment shows satisfactorily that the sugar which disappears under the action of insulin is not converted into fat in the liver.

We are greatly indebted to Dr H. H. Dale for valuable help and advice during the course of these experiments.

Since this paper was written we have learned that Professor Babkin, working independently at University College, has been studying the same problem, and that his results are in agreement with ours.

Summary.

1. The sugar which disappears from the blood of normal animals under the influence of insulin is neither converted into nor stored as glycogen either in the liver or in the skeletal muscles.

2. The glycogen of both liver and skeletal muscle disappears almost completely in animals which are given a dose of insulin sufficient to cause hypoglycaemic convulsions.

3. There is no evidence of a conversion of carbohydrate into fat in normal animals under the influence of insulin.

References.

Abderhalden (1914). Text Book of Physiol. Chem. (Hall), 48.
Banting and others (1922). *Trans. Roy. Soc. Canada*, **16**.
Dale (1923). *Lancet*, i, 989.
Dudley (1923). *Biochem. J.* **17**, 376
Dudley, Laidlaw, Boock and Trevan (1923). *J. Physiol.* **57**; *Proc. Physiol. Soc.* March.
Kellaway and Hughes (1923). *Brit. Med. J.* i, 710.
Shaffer and Hartmann (1920). *J. Biol. Chem.* **45**, 365.